# Olfaction and scent discrimination in African elephants

# (*Loxodonta africana*)

by

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# Declaration

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This dissertation includes one original paper published in a peer-reviewed journal and four unpublished publications, one of which is currently under review. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: 1 March 2021

# Summary

As an endangered mammal, novel research into the African elephant's ethochemistry will assist ethologists, conservation biologists and wildlife managers in understanding odour signals in elephant exudates, and how they impact elephant behaviour in captivity and in the wild. Analytical methods combining genetics, statistics with SPME GC_MS and innovative metabolomics visualisation software are a useful first step in unlocking odours salient to elephant reproduction, breeding and behaviour. Mammals, such as the African elephant, which live in sophisticated, fission-fusion, social systems marked by linear dominance hierarchies and ritualized greeting ceremonies, require an effective means of communication in order to maintain social cohesion. A number of acoustic, olfactory and visual signals have been described. Within a group context, individuals need to discriminate between members of their group and foreign conspecifics in order to avoid inbreeding and to identify recipients of nepotistic behaviour. Such olfactory group/herd/clan/colony identity signals have been described in a number of mammals such as beavers, bats, naked mole rats, rabbits, fur seals, lemurs and hyena but are lacking in the iconic African elephant. It is to date not clear what fixed and variable information is encoded in African elephant urine, temporal gland secretions (TGS), genital and buccal secretions, and whether an olfactory signal for herd membership exists, although scientists have long expected this to be the case. Specifically, African elephant TGS, buccal and genital secretions in adult females have not been described in a free-ranging population. The overall objective of this research was to augment scientific understanding of African elephant olfactory behaviour in a multi-disciplinary way, combining behavioural observation and experimental trials of African elephants in sanctuaries in South Africa, with genetic and chemical data sampled from a free-ranging African elephant population during a translocation event in Malawi. The research first assesses olfactory acuity and scent discrimination ability in African elephants through novel behavioural bioassays. Subsequently, this study examines whether chemical signals in urine, TGS, buccal and genital secretions encode for age, sex and identity, and explores whether odour is correlated with genetic relatedness. For the first time, the chemistry in TGS, buccal and genital secretions is characterised in wild African elephant adult females (n=40).

Elephants were found to be highly adept at scent-discriminating between humans, and scent-tracking a target human across various substrates, including distractor trails. Results suggest that elephants may utilise olfaction to discriminate between individual conspecifics. This was tested using behavioural bioassays, which concluded that African elephants can discriminate between unfamiliar and familiar conspecifics from both urine and dung. Urine elicited the most interest, and was used for further detailed analysis using the comprehensive metabolomics tool XCMS online. TGS, buccal and genital secretions from wild-sampled elephants (n=113) were found to encode individual identity in African elephants. TGS and buccal secretions encoded for age, and TGS and genital secretions tended towards significance for sex. Genetic relatedness was not correlated with odour in TGS, buccal and genital secretions at the herd level. However in all three odours, Similarity Percentage Analysis (SIMPER) identified "herd" as a highly significant factor in explaining chemical differences between herds. This suggests that group odour in African elephants is not correlated to genetic relatedness at the population, herd and individual level, and allows for the possibility that bacteria may be involved in group odour. The biomarkers alkan-2-ones and alkan-2-ols previously published in association with elephant male urinary microbial communities, were identified in adult female buccal and labial secretions. SIMPER analyses further extracted a number of short-chain fatty acids significant to chemical differences between groups (notably acetic acid, pentanoic acid, hexanoic acid, decanoic acid,

dodecanoic acid and tetradecanoic acid). These and other compounds have been identified in similar studies as key volatile compounds from bacterial fermentation in clan-living hyenas and colonially-living meerkats, further supporting this possibility. The frequent affiliative social behaviour of African elephants is posited as a likely mechanism for bacterial transmission. Conservation objectives can be supported by an improved understanding of how chemical communication in social mammals like elephants influences mate choice, establishing social relationships, courting, mating and the use of scent for establishing territories. In African elephants, an improved understanding of elephant semiochemistry and eliciting behaviour could shed light on scent manipulation for elephant translocation and reintroductions, captive breeding, welfare and enrichment protocols, human elephant conflict (HEC), elephant reintroductions, social behaviour and habitat selection.

# Opsomming

Vir 'n bedreigde soogdier, sal nuwe navorsing oor die etochemie van die Afrika-olifant, etoloë, bewaringsbioloë en natuurlewebestuurders help om reukspore in olifante-eksudate te verstaan, en hoe sulke -spore die gedrag van olifante in aanhouding en in die natuur kan beïnvloed. Analitiese benaderings wat genetika, statistieke met vaste fase mikro-ekstraksie gevolg deur gaschromatografie-massaspektrometrie (SPME-GC-MS) en innoverende sagteware vir visualisering van metabolomika kombineer, is 'n nuttige eerste stap in die ontsluiting van reukspore wat opvallend is vir reproduksie, teling en gedrag van olifante. Soogdiere, soos die Afrika-olifant (AO), wat in gesofistikeerde sosiale stelsels leef wat gekenmerk word deur lineêre dominansie hiërargieë en geritualiseerde groetplegtighede, benodig 'n effektiewe kommunikasiemiddel om sosiale kohesie te handhaaf. 'n Aantal akoestiese, reuk- en visuele spore is beskryf. Binne groepsverband moet individue onderskei tussen lede van hul groep en vreemde lede om inteling te vermy en om ontvangers van nepotistiese gedrag te identifiseer. Sulke reukspore vir groep/kudde/stam/kolonie identiteitsseine is beskryf in 'n aantal soogdiere soos bewers, vlermuise, naakte molrotte, konyne, pelsrobbe, lemurs en hiënas, maar dit ontbreek vir die ikoniese AO. Dit is tot dusver nie duidelik watter vaste en veranderlike inligting in AO-uriene, temporale klier- (TGS), geslags- en bukkale afskeidings gekodeer is nie. Meer spesifiek, AO TGS, bukkale- en geslagsafskeidings by volwasse olifantkoeie is nie in 'n vrylopende AO-populasie gekwantifiseer nie. Dit is ook onduidelik of daar 'n reukspoor vir kuddelidmaatskap bestaan, hoewel wetenskaplikes dit al lank vermoed het.

Die oorkoepelende doel van hierdie navorsing was om die wetenskaplike begrip van die olifaktoriese gedrag van Afrika-olifante te verbeter deur 'n multidissiplinêre benadering te gebruik, wat gedragswaarneming en eksperimentele proewe met Afrika olifante in toevlugsoorde in Suid-Afrika kombineer, met genetiese en chemiese gegewens uit 'n vrylopende olifantpopulasie tydens 'n translokasie-geleentheid in Malawi. Die navorsing beoordeel eers reukskerpte en reukdiskriminasievermoë by Afrika-olifante deur middel van nuwe gedrag bio-bepalingsmetodes. Vervolgens word hierdie studie ondersoek of chemiese seine in uriene, TGS, bukkale- en geslagsafskeidings kodeer vir ouderdom, geslag en identiteit; en daar was ook ondersoek of reuk verband hou met genetiese verwantskap. Vir die eerste keer is die chemiese aspekte in TGS, bukkale- en geslagsafskeidings in 41 wilde volwasse AE-wyfies gekenmerk.

Daar is gevind dat olifante hoogs vaardig is in die reuk-onderskeiding tussen mense en die reuk-opsporing van 'n teikenmens oor verskillende soorte substrate, insluitend afleidingspore. Resultate dui daarop dat olifante olfaksie kan gebruik om onderskeid tussen gelyksoortige individue te onderskei. Dit is getoets aan die hand van gedrag biobepalings, wat tot die gevolgtrekking gekom het dat Afrika-olifante onderskeid kan tref tussen onbekende en bekende gelyksoortige individue deur beide uriene en mis. Uriene het die meeste belangstelling ontlok en is gebruik vir verdere gedetailleerde ontledings met behulp van die omvattende aanlyn metabolomika-instrument XCMS. Analises van TGS, bukkale- en genitale afskeidings van 113 wilde AO het aangedui dat kodering vir individuele identiteit in AO voorkom. Analises het aangedui dat afskeidings van die temporale en bukkale kliere wat vir ouderdom gekodeer is, en TGS en genitale afskeidings geneig is om te kodeer vir geslag. Genetiese verwantskap was nie gekorreleer met reuk in TGS, bukkale- en geslagsafskeidings op kuddevlak nie. In al drie reuke, het 'n soortgelyke persentasie-analise (SIMPER) 'kudde' geïdentifiseer as 'n baie belangrike

faktor om chemiese verskille tussen kuddes te verklaar. Dit dui daarop dat groepsreuk by Afrika-olifante nie gekoppel is aan genetiese verwantskap op populasie-, kudde- en individuele vlak nie en dit moontlik maak dat bakterieë by groepsreuk betrokke kan wees. Die biomerkers alkan-2-ene en alkan-2-ols, wat voorheen gepubliseer is ten opsigte van olifantbul urien mikrobiese gemeenskappe, is geïdentifiseer in volwasse vroulike bukkale en labiale afskeidings. SIMPER-ontledings het 'n aantal kortkettingvetsure wat veral chemiese verskille tussen groepe verklaar (veral asynsuur, pentansuur, heksaansuur, dekanoësuur, dodekanoësuur en tetradekanoïensuur) onttrek. Hierdie en ander verbindings is in soortgelyke studies geïdentifiseer as die belangrikste vlugtige verbindings van bakteriële fermentasie in stamlewende hiënas en koloniale lewende meerkatte, wat hierdie moontlikheid verder ondersteun. Die gereelde affiliatiewe sosiale gedrag van AO word beskou as 'n waarskynlike meganisme vir bakteriële oordrag. Bewaringsdoelstellings kan ondersteun word deur 'n beter begrip van hoe chemiese kommunikasie by sosiale soogdiere soos olifante, die keuse van die maat, vestiging van sosiale verhoudings, hofmaak, paring en die gebruik van reuk vir die vestiging van gebiede, beïnvloed. By Afrikaanse olifante is 'n beter begrip van olifant semio-chemie en verwante gedrag kan hulp verleen met die verskuiwing en herbekendstelling van olifante, teling in aanhouding, welsyn- en verykingsprotokolle, bestuur van mens-olifant konflik, sosiale gedrag en kies van habitat.

# Dedication

This dissertation is dedicated to my children Valentina and Cuno:
may your inquiring minds forever marvel at the wonders of our natural world.

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# Notes

This thesis is presented in the format prescribed by the Department of Conservation Ecology, Stellenbosch University. This thesis is a compilation of individual chapters and some degree of repetition is inevitable.

Referencing is in accordance to the journal guidelines for chapters that were published (Chapter 2) and submitted for review (Chapter 3).

In order to improve our understanding of specifically African elephant semiochemicals, and female elephant signaling in particular, this study focused on broadly characterizing semiochemicals in African elephant urinary, temporal, buccal and genital secretions, with a specific focus on female chemical signaling. Adult female semiochemistry remains to be characterized from a significant sample size, as prior research has been constrained by small sample sizes restricted to individuals in non-native, captive settings. Verifying chemical compounds through the addition of standards was considered unimportant to this exploratory study, however it is the requisite next step before testing behavioural function of selected compounds in the field through behavioural bioassays.

Due to the exploratory and novel nature of the research, Chapters 5 and 6 were kept separate and semiochemicals listed within chapters for ease of reference, however for publication purposes, these chapters shall be combined.

Graphics automatically generated by specialized software programs such as XCMS and Agilent Technologies (chromatograms) cannot be formatted to University standards, and were consequently left in their original format.

# Nomenclature

| | |
|---|---|
| **AP** | **African Parks** |
| **AWE** | **Adventures with Elephants** |
| **AWT** | **Africa Wildlife Tracking** |
| **DNA** | **Deoxyribonucleic acid** |
| **HEC** | **Human Elephant Conflict** |
| **EW** | **Elephant Whispers** |
| **GC-MS** | **Gas Chromatography/Mass Spectrometry** |
| **GNRH** | **Gonadotropin Releasing Hormone** |
| **LMW** | **Low Molecular Weight** |
| **MDS** | **Multidimensional Scaling** |
| **MHC** | **Major Histocompatability Complex** |
| **MTS** | **Matching to Sample** |
| **MW** | **Molecular Weight** |
| **MWR** | **Majete Wildlife Reserve** |
| **MUP** | **Major Urinary Protein** |
| **NIST** | **National Institute of Standards and Technology** |
| **NWR** | **Nkhotakhota Wildlife Reserve** |
| **OR** | **Olfactory Receptor** |
| **QG** | **Queller Goodnight** |
| **RHCRU** | **Rory Hensman Conservation and Research Unit** |
| **SCFA** | **Short Chain Fatty Acid** |
| **SPME** | **Solid Phase micro extraction** |
| **SWGDG** | **Scientific Working Group on dog and orthogonal detector guidelines** |
| **TIC** | **Total Ion Chromatogram** |
| **TFCA** | **Transfrontier Conservation Area** |
| **TGS** | **Temporal Gland Secretions** |
| **VNO** | **Vomeronasal Organ** |
| **VOC** | **Volatile Organic Compound** |
| **VP** | **Vapour Pressure** |
| **XCMS** | **Extended Content Management Suite** |

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# Chapter 1: The role of chemosignalling in elephant conservation

*"The sense of smell is of the highest importance to the greater number of mammals ….[]"*
*Charles Darwin (1871)*

## 1.1 Background and objectives

The threat to elephant populations and the decline in elephant numbers and range in Africa due to human population growth, human elephant conflict and poaching are recognized as the continent's most serious conservation challenges (Biggs et al. 2017, Gray & Gauntlett 2017, Masanja 2014). The current elephant population in Africa is estimated to stand at ~400 000, with >70% of elephants occurring in southern Africa (CITES 2017). Although poaching in Africa may have declined by 7% since 2011 (Hauenstein et al. 2019), the species remain at risk (Schlossberg et al. 2020): human population growth in Africa, estimated to double by 2050 to 2.5bn people (The Economist 2020), is a significant threat to available elephant range land, with poverty-fuelled deforestation and land transformation to agriculture a direct threat to the environmental stability in the region (Bradshaw & Minnin 2019). Importantly, funding for wildlife management in Africa has been severely compounded by the current - and persistent – impacts of the global Covid-19 pandemic, with shortfalls in conservation revenue having been exacerbated by the lack of tourism income. Funding for park management and anti-poaching units has been severely compromised, with a significant rise in poaching of elephant and other wildlife (Lindsay et al. 2020).

As iconic megaherbivores, African elephants are a keystone species in African ecosystems as they impact vegetation structure and abundance (Cumming et al. 1997) and consequently the presence and absence of other species (Pringle 2008). The loss of elephants from any ecosystem impacts ecosystem processes with effects on vegetation, insects, birds and other mammals (Midgley et al. 2020, Haynes 2012). A comprehensive meta-analytical study evaluating elephant impact on ecosystems across the continent established that elephants contribute positively to the maintenance of African savannas (Guldemond et al. 2017).

The African elephant consequently requires global and multi-disciplinary efforts to sustain its populations, conserve rangeland and manage its interactions with people if it is to continue to

survive and thrive in the next century. Conservation strategies include rangeland management in open systems that promote dispersal and outbreeding, for example in transfrontier conservation areas (TFCAs). Management of such large landscapes includes the designation of wildlife specific corridors, buffer zones and stepping stones in an increasingly anthropogenic matrix, as well as Human Elephant Conflict mitigation and education of the rural public (Shaffer 2019, von Dürckheim 2011, von Dürckheim et al. 2012, Hanks 2003). In fenced environments, translocation, culling and contraception are used to manage elephant density and impact on biodiversity (Selier et al. 2018). Elephants require hands-on active management in smaller zoos and sanctuary environments, yet North American populations of African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants are declining and captive breeding is historically poor. According to Wiese (2000), without continued importation or a drastic increase in birth rates, the Asian elephant population in North America will drop to approximately 10 elephants in 50 years and be demographically extinct. An improved understanding of factors underlying elephant behaviour in both natural and captive settings can contribute towards improved conservation outcomes.

Behavioural interaction between conspecifics is governed by intraspecific chemical signalling. It is argued that, consequently, chemical communication is possibly the most important mode of communication in mammals (Wyatt 2003, 2009). Unlike auditory or visual signals that are short-lived and transmit an immediate message to a receiver, olfactory signposts such as dung or urine spots remain in the environment for longer. Chemical communication is linked to reproduction, mate choice and attraction, territoriality, parental care, kin discrimination and disease transmission, all of which inform population dynamics and structure. Chemical signals encode age, rank and sex of individuals, for example in mandrills (Setchell et al. 2010), and rhinos (Marneweck et al. 2017), and encode genetic differences between species and subspecies (Heth et al. 2003, Busquet & Baudoin 2005). At a fine scale, research has revealed that chemical signals encode information about genetic quality and relatedness in fur seals (Stoffel et al. 2015) and lemurs (Charpentier et al. 2008). Kean et al. (2017) found that otters possess chemical signals for sex and biogeography-specific odours. Chemical signals have been significantly correlated to clan/group/colony identity in bats (Bloss

et al. 2002), fur seals (Stoffel et al. 2015), chimpanzees (Henkel & Setchell 2018), rhesus macaques (Henkel et al. 2015) and to individual identity in hyenas (Burgener at al. 2009) and raccoons (Kent & Tang-Martinez 2014). Chemical expression of genetic relatedness in an individual has been proposed to promote outbreeding, facilitate nepotism and function in phenotype matching, kin discrimination (Boulet et al. 2009) and mate choice. A number of studies have explored the correlation between individual phenotype and MHC genotype (Wedekind et al. 1995, Penn & Potts 1998).

Elephants have numerous morphological adaptations to support chemical communication. Elephants have the largest absolute brain in terrestrial mammals, with a large amount of cerebral cortex, especially in the temporal lobe, and a well-developed olfactory system comprised of a main olfactory system, vomeronasal system and palatal pits. Neuroanatomical studies indicate a highly convoluted cerebral cortex and a series of 32 large olfactory turbinals, primary endoturbinals (Rasmussen 1998) and an unusually extensive bilobed vomeronasal organ (VNO), with a large rhinencephalon, or olfactory bulb. These structures are linked to complex learning and behavioural functions in humans, while elephant brain size itself may be associated with memory storage and intelligence (Shoshani et al. 2006). The enlarged neocortex is shared by other species such as humans, apes and certain dolphins; species that have been attributed with higher intelligence and cognitive abilities. Elephants emit chemical signals in urine, dung, toes, ears and genitalia. Despite the obvious sexual dimorphism in the species, African elephants of both sexes have a highly specialized apocrine scent gland, the temporal gland. Notably, the inside back legs and face of breeding elephant bulls are stained during periods of musth (a rut-like period where adult males seek out receptive females and constantly dribble urine), while females faces are stained by temporal gland secretions during ritualized greeting ceremonies, when excited or distressed. Elephants, a widely spatially dispersed species, engage in scent marking through depositing urine, dung and temporal gland secretions (TGS) along pathways, at waterholes, and on vegetation, which elicits significant interest from sexually mature male and female conspecifics (Merte et al. 2010). During contact periods and social events, elephants excrete body odour such as urine, TGS, and dung during greeting ceremonies, while genitalia and mouth odour are extensively sniffed and evaluated by

both sexes and across age classes. Olfactory communication is key to elephant social interactions as witnessed by their olfactory behaviour, unique proboscis, olfactory system, large number of olfactory receptor genes (Niimura et al. 2014), elaborate scent marking during greeting ceremonies and the intensity of responses that scent elicits from conspecifics.

Understanding elephant communication and behaviour consequently contributes towards this species conservation success (Schulte et al. 2007, Stoeger et al. 2014, Viljoen et al. 2009). The importance of specifically chemical signals to elephants was recognized in early Sanskrit writing: “Upon smelling their own dung and urine, let them always be producing a tickling of the palate (Ch1, Stanza 1”), and […] “those who are born in spring are scent elephants. And from the smell of their sweat, dung, urine and must-fluid, other elephants are instantly excited” (Ch1, Stanza 38).

As promulgated by Darwin’s theory of sexual selection (Darwin 1871), chemosensory research in mammals to date has explored male sexual signalling and its role in attracting conspecific females and mediating competition between rival males (Clutton-Brock & McAuliffe 2009). This has also been the case with chemical research in elephants, which has primarily focused on captive Asian elephants and sexual signalling in captive Asian bulls in musth (Goodwin et al. 2012, Schulte & Rasmussen 1999, Rasmussen 1998, 2000, Rasmussen & Perrin 1999, Greenwood et al. 2005), probably due to male-biased sexual dimorphism and scent-signalling behaviour in Asian elephants. Endocrinological, chemical and behavioural research on African elephant bulls have evaluated musth in TGS and urine (Ganswindt et al. 2005). However, female mammals also use scent signalling for sexual attraction, mediation of female competition and cooperation, and to facilitate maternal behaviour (Clutton-Brock & Huchard 2013). Chemical signalling, and how it governs behaviour and sociality is particularly interesting in African elephants, as females are philopatric, live in matrilineal, multi-tiered, fission-fusion groups, marked by linear dominance hierarchies, reciprocal cooperation, coalitions, and differentiated social relationships between group members which are indicative of social complexity (Lukas & Clutton-Brock 2018). Like males, females possess a specialized scent gland, the temporal gland, and engage in ritualized greeting ceremonies with conspecifics where TGS is secreted, in addition to urine and dung. The function of these secretions, and how

they relate to social signalling among African elephant females, is poorly understood and to date, research on African elephant female chemical communication has been limited, and constrained by sample size.

*Sexual signalling*: Numerous studies on Asian elephants have shown that the chemistry in elephant bulls' TGS are correlated with fluctuating testosterone levels. Musth is expressed in Asian and African bull TGS and urine, and younger males avoid males in musth. Olfactory displays alerts conspecific males to the condition of musth, with males secreting from the temporal gland and dribbling urine constantly from an unsheathed penis. Such visual and olfactory displays reflect the signallers hormonal, status, condition and physical strength (Clutton-Brock & Huchard 2013). Research on elevated testosterone levels in Asian and African elephant males suggests that musth contributes towards the "smooth functioning of male elephant society" as musth males are dominant over non-musth males, and musth in bigger, older bulls suppresses reproduction (musth) and aggression in younger males (Slotow et al. 2000), with chemosignals in musth varying with age, dominance and reproductive status (Greenwood et al. 2005). Male African elephants can distinguish conspecific female urine collected at the time of ovulation from urine obtained at the mid-luteal time of the estrous cycle (Bagley et al. 2006). Males detect females in oestrus, and respond with flehmen, penile erections and premature mating behaviour (Rasmussen et al. 1996, Rasmussen et al. 1997). The most extensive study on African elephants demonstrates inbreeding avoidance in African elephants, where bulls not only avoid breeding with natal kin but also with paternal kin (Archie et al. 2007), suggesting that males may be using phenotype matching to identify kin.

Female chemical signals advertise sexual receptivity and fertility in both Asian and African elephants, with chemical and behavioural studies identifying pheromones suggesting impending ovulation in Asian elephant urine, and pheromone presence of the same in African elephants. This inter-sexual signalling is important in spatially flexible species such as elephants, where females must attract mates from a distance. As is the case in numerous mammals such as lemurs (Drea & Scordato 2008), pandas (Swaisgood et al. 1999) and hamsters (Huck et al. 1989), male elephants monitor the receptivity in female elephants' urine. A study by Meyer, et al. (2008) found that female elephants monitor conspecific females' oestrus cycle by sniffing

urine and checking the genitalia of conspecifics, but it is not known if an oestrus pheromone is expressed in labial secretions.

*Social signalling*: Olfactory signals are suggested to play a role in mediating both cooperative and competitive interactions between females. Whereas males compete for females, females compete for access to resources (Clutton-Brock et al. 1988, 1989). As elephant females invest heavily into gestation and lactation, reproductive success is constrained by the direct availability of resources and access to food. In fact, studies on effects of resource competition among females has shown the negative effects of increasing group size, which was found to depress fecundity and increase mortality in females and their offspring (Clutton-Brock et al. 2008). In group-living species, females are found to compete with other females in having and raising offspring. Competitive female relationships are determined by linear dominance hierarchies in a number of social species, with dominant females regulating conflict and subordinate species avoiding dominant conspecifics. In African elephants, dominance hierarchies among females emerge in the dry season, in response to resource constraints such as water and food (Wittemyer et al. 2007). This is also the case in certain primates, where clear linear dominance hierarchies among females are associated with access to patchily distributed food resources (Bernstein 1981). Dominance is further linked to reproductive success in hyena (East 1993) and to preferred access to resources and higher reproductive output (Holand et al. 2004), with consequent higher offspring survival. Dominance in highly ranked females is also linked to access to superior males and paternal care, and to reduced risk of predation and improved survival (Silk et al. 2010). Dominance rank has been correlated to age and size in African elephant (Archie et al. 2006), meerkats (Hodge et al. 2008), chimpanzees (Pusey et al. 1997) and dolphins (Samuels & Gifford 1997). Kinship Selection Theory (Hamilton 1964) posits that competition between group-living related individuals should be minimal, with closely related individuals displaying behavioural tolerance and support for kin. In undisturbed populations, African elephant herds are comprised of highly related individuals, with fission and fusion among herds more likely if the matriarchs are maternal sisters. However in disturbed populations, kinship is not a predictor of fission-fusion dynamics. It has been shown that orphaned elephants joining new families are subordinate to the dominant females, spending

affiliative time with age mates and subordinate females. This lack of affiliation with dominant adult females suggests that orphans may experience decreased resource access and associated fitness cost (Goldenberg & Wittemyer 2017). Rank dependent and other supportive relationships among females are found in matrilineal and other societies, where the social support of conspecific females is associated with the formation of large, stable groups comprising multiple breeding females which may be related or not. According to Clutton-Brock (2009a), the effects of social support of female dominance and fitness in itself has resulted in the development of complex affiliative relationships. Odour allows for recognition of group members or cooperative partners, which is important in social species such as elephants that raise their offspring together. Odours in elephants have been suggested to promote cohesion and stability in elephant bull groups (Rasmussen & Greenwood 2003) as well as in female herds (Wyatt 2003). Advertising group membership is important in fission-fusion societies, where core units can separate from matrilineal clans or bond groups for weeks or months during the dry season, and fuse again when resources are abundant. Group odour has been identified in bats, spotted hyenas, badgers and meerkats (Bloss et al. 2002, Burgener et al. 2008, Gorman et al. 1984, Leclaire et al. 2017). Studies on primates and rodents suggest that group odour is correlated to genetic pairwise relatedness (Boulet et al. 2009, Tzur et al. 2009). On the other hand, evidence from other studies proposes that group odour may be caused by differences in bacterial communities between groups (Leclaire et al. 2017, Archie & Theis 2011). Studies on mate choice and kin recognition in humans, hamsters and badgers implicate Major Urinary Proteins (MUP) and the highly polymorphic Major Histocompatability Complex (MHC) set of genes in olfactory signals of genetic relatedness (Hurst et al. 2002, Wedekind & Penn 2000). The level of olfactory similarity among individuals within elephant herds, across herds and individuals, and the influence of genetic and other factors such as physiology, diet and bacteria on chemical profiles, is hitherto unknown in elephants, both Asian and African. Given the paucity of information on adult African female semiochemicals and their role in elephant female society, a chemical study on adult female semiochemicals would elucidate semiochemical functional roles and behaviour during elephant ritualized greeting ceremonies, in parent-offspring recognition, fission-fusion dynamics, mate choice, female dominance

hierarchies, kin recognition and social cohesion. Research on semiochemicals of specifically African elephant females TGS, is notably absent from the scientific literature, as unlike their Asian counterparts, African elephant female adults secrete TGS frequently, particularly during social interactions. Similarly, semiochemical characterization of genital and buccal secretions are absent from the literature, which is perplexing given the importance of both the mouth and genitalia in intra and inter group olfactory behaviour in elephants (Schulte et al. 2007). Importantly, the secretion of TGS, urine and dung by female African elephants during ritualized greeting ceremonies between herds during fusion begs the question as to what these secretions, excretions and body odours encode, and why females fan their ears and spin their bodies in the process. This behaviour suggests that elephant females are signalling a pachyderm perfume and much like Lepidoptera that use the fanning of wings to spread pheromones, elephant females do the same during greeting ceremonies by fanning their ears. What the signals contain is a mystery, but, given what is known about male TGS, female TGS may likely encode a chemical cue that promotes group cohesion, which can serve to reduce conflict among group-living mammals.

While elephants use auditory, behavioural and olfactory modalities to communicate with proximate conspecifics, chemical cues are likely to be the more important source for honest signals related to fixed (gender, identity) and variable factors (Schulte et al. 2007) for communication with spatially distant conspecifics. Especially in a socially and spatially flexible species such as the African elephant, understanding the information contained in chemical cues in urine or dung is important. Urine and dung, which are olfactory signposts left at waterholes and on elephant pathways (von Dürckheim et al. 2012), are likely to contain persistent scent marks relating to sex, reproductive state and genetic relatedness. It is expected that these scent marks would contain heavier molecules with low vapour pressure, such as the stable aromatic compounds that signal dominance and territory. For example, certain compounds in scent signals appear to get stronger after deposition, such as exo-brevicomin in mouse urine (Apps, et al. 2015), and some fade out after 1-2 days. Chemical signatures have been shown to increase in intensity over time (Goodwin et al. 2006), and certain urinary signals may last 40 days or longer as is the case in dwarf mongoose anal sac secretions. The longevity of a signal

may be attributable to high molecular weight (MW) compounds and or to the presence of fixatives that retard the emission of low molecular weight compounds. Elephant TGS is known to contain high MW proteins, lipids and steroids, and urea in mammalian urine is suspected to function as a fixative enabling long-lasting scent signalling from urine. Over three days at room temperature, the concentration of volatile ketones, alcohols, and alkyl phenols in both African and Asian bulls' urine increases (Goodwin et al. 2006), with strengthened pheromonal signalling of exo-brevicomin and frontalin. In elephant urine, the ketone, alcohol and protein- derived aromatic metabolites increased with the age of the urine, due to possible microbial metabolism of fatty acid (Goodwin et al. 2016). The pungent odour of elephant musth in TGS and urine are derived from bacterial metabolisation of fatty acids (Goodwin et al. 2016). Microbes consequently appear to play a significant role in some of the characteristic odourants in elephants.

Musth bulls wander great distances in pursuit of receptive females, as females are receptive for one week every four to five years depending on length of calving intervals. Bulls dribble urine constantly along elephant pathways, and deposit musth TGS on to branches and twigs by rubbing their heads against the vegetation – these musth scent marks alert receptive females to his reproductive status, and other males to his competitive presence. Bulls loose significant body condition during this time, and cannot afford to invest energy into tracking unreceptive or related females. Studies have confirmed that urine contains chemical cues for breeding condition and impending ovulation in Asian elephants – frontalin in male urine, and (Z)-7-dodecen-1-yl acetate in female oestrus urine. While the presence of frontalin has been confirmed in African elephants, the female oestrus pheromone is known to exist but remains to be identified (Meyer et al. 2008). In socially complex species, females benefit from recognizing conspecifics that belong to their clan or bond group, or identifying unfamiliar, dominant females in order to avoid competition and conflict in the dry season when diminishing resources constrain the benefits of sociality. Elephant females monitor the location of familiar and related females of greater clan or bond groups through urinary scent marks and are able to recognize up to 30 family members from their urine (Bates et al 2008). It is consequently likely that excretions such as urine, dung and glandular secretions such as TGS, deposited by signallers

in the absence of conspecifics, function as long-lasting scent marks, which encode individual identity, reproductive status and quality.

Emanating body odour, as opposed to scent marks such as buccal or labial secretions, are further olfactory modalities that may encode reproductive and social odours (Drea 2014). Aldehydes, which are prone to oxidation, are more common in body odours (Apps et al. 2015). Elephant body odour is consequently likely to signal socially relevant information of immediate importance regarding fixed (gender, identity) and variable factors (age, rank, health, reproductive state) during short-term behavioural interactions such as during greeting ceremonies between herds, and encounters between males. According to Apps et al. (2015), complex sociality based on individual identity requires a wide range of social signals. Some studies suggest that only molecules with high MW can be considered to be functional as signals in mammalian chemical communication, while others purport that only compounds of mammalian origin are significant (Charpentier et al. 2012). However, signal components do not have to be animal metabolites. This is supported by Archie and Theis (2011), who argue that group odour may be generated by shared bacteria.

In conclusion, chemical signalling is central to social recognition, territory control, reproduction, and alarm signalling across Mammalia (Wyatt 2014). Improved fundamental knowledge of the composition and the possible function of semiochemicals in African elephant behaviour is needed, as this knowledge would advance conservation and management efforts by improving our understanding of elephant behaviour.

**1.2 Semiochemicals and conservation:**

The use of semiochemicals in conservation models is not new. Semiochemical research has been extensively applied to insect conservation and pest management. The identification of pheromones and kairomones as sex attractant and aggregation pheromones has yielded a plethora of new conservation management models for insects across the globe, both in protected areas and agricultural and forestry land (Smart et al. 2014). Insect semiochemicals have been applied in a diversity of conservation contexts such as conservation monitoring, population monitoring and distribution, movement and dispersal changes, landscape and habitat interactions (Larsson 2016).

Conservation objectives can be supported by an improved understanding of how chemical communication in social mammals like elephants influences mate choice, establishing social relationships, courting, mating and the use of scent for establishing territories. Especially with regards to management of wild populations (Mueller–Schwarze & Heckman 1980), successful captive breeding efforts of endangered mammals (Lindburg & Millard 1997, Kleiman 1994) and reintroductions into the wild, understanding how chemical communication within species governs social and sexual signalling becomes important. In African elephants, an improved understanding of semiochemistry could shed light on scent manipulation for elephant translocation and reintroductions, captive breeding, welfare and enrichment protocols, human elephant conflict (HEC), elephant reintroductions, social behaviour and habitat selection. In African elephants, semiochemical research may be applied to the conservation of the species as follows:

1. Population monitoring through use of urine and dung samples at scent marking sites such as waterholes and elephant pathways, could provide information on identity, status, age, sex of an individual and monitor population densities, especially in secretive elephant populations. Research suggests that elephant urine may further contain genetically programmed constituents reflective of relatedness (Rasmussen & Krishnamurthy 2000), and if proven, could prove a useful and non-invasive way of population monitoring.

2. HEC and identification of culprits – recognition and monitoring of known individuals can contribute towards the identification and management of problem elephants and repeat

offenders. Young male Asian elephants for example, show avoidance behaviour to the TGS scent of musth bulls, retreating from the signal (Rasmussen & Krishnamurthy 2000). This is significant as crop-raiding African elephants tend to be young males (von Dürckheim 2011).

3. Habitat selection – the cues that elephant use to navigate a foreign landscape post relocation or translocation is of interest. The presence of familiar conspecifics is significant (Stamps 1988). Elephants deposit scent marks such as urine and dung along pathways, and an improved understanding of what these scent marks encode could be used to manipulate habitat selection and aid in promoting relocation success.

4. Captive welfare - olfactory enrichment can be an important tool in captive animals. Captive breeding programs could benefit from an improved understanding of how odour governs social cohesion and limits competition among conspecifics. Odours in urine, dung or breath, signalling impending ovulation, musth, pregnancy, dominance, or stress, could provide non-invasive tool to manage a captive population.

5. Captive breeding: The introduction of individuals into an established population can result in aggression and/or stress, or initiate physiological response in terms of reproduction, receptivity, and dominance. Further, scent marks containing relatedness markers could be used to monitor levels of hybridization and inbreeding,

6. Translocation and relocation programs – semiochemicals that promote social stability are of interest. One study showed that the presence of old mature musth bulls controls the behaviour of younger rogue males (Slotow et al. 2000). It is as of yet unclear whether semiochemicals in female exudates provide a similar function.

7. Health status and disease – stress in the form of salivary Immunoglobin A, fecal glucocorticoid metabolite (fGCM) levels can be monitored in captive and free-ranging elephants in order to manage stress in closed small systems such as sanctuaries and zoos, as well as in TFCAs in terms of landscape planning and human disturbance. Research into early detection methods for tuberculosis, haemorrhagic septicaemia, trypanosomiasis, pyroplasmosis, foot and mouth disease, pox, bacillary necrosis, salmonellosis, streptococcosis, babesiosis, helminthiasis and ectoparasitism, rabies, tetanus, Anthrax, and Elephant endotheliotropic herpesviruses (EEHV), and their link to expression in elephant odour, would be of value.

### 1.3 Aims and objectives:

The overarching aim of this PhD is to improve our understanding of African elephant olfaction and behaviour. Olfactory and scent discrimination ability in African elephants was evaluated through the development of novel behavioural bioassays in a captive population. The specific objectives of this study were to 1) describe the semiochemicals in African elephant temporal, genital and buccal secretions 2) to assess what fixed (sex, identity, relatedness) and variable (age) information is encoded in the odours and 3) to test whether elephant families share a similar olfactory signal. To date, no research exists on Odour-Gene Covariance (OGC) in African elephants, and nothing has been published on African elephant female TGS, nor on the semiochemistry of buccal and genital odour in free-ranging populations of African elephants. A combination of statistical, genetic, Gas Chromatography Mass Spectrometry (GC_MS) and metabolomics visualization tools were applied to elucidate these.

### 1.4 Dissertation Structure:

Chapter 1 introduces the study and provides a background to olfaction in elephants and the importance of semiochemicals to elephant behaviour and conservation. Chapter 2 explores olfactory acuity in African elephants by evaluating scent discrimination of human VOCs. The results show that African elephants can distinguish between nine different humans, spanning three generations, based on skin VOCs. A further study explores elephant ability to track an odour-trail to a food source (Chapter 3). Results here showed that elephants can track a human odour trail one hour after it was laid to a food source, and the animals discriminated between trails laid by different humans (original and distractor). The results made me question whether elephants can discriminate between familiar and unfamiliar conspecifics. Subsequently in Chapter 4, tests whether elephants can distinguish between conspecific familiar and unfamiliar urine and dung were conducted. Results reveal that elephants are able to distinguish between the urine and dung of conspecifics, and that individuals spent more time investigating conspecific urine. A novel metabolomics cloud-based chemometric analysis was then applied to samples of elephant urine, looking for olfactory signals for sex and age. A translocation event (www.500elephants.org) provided an opportunity to sample 113 elephants for temporal, genital

and buccal secretions. These were analyzed in conjunction with individual genotype data to test test whether exudates encoded for relatedness, identity, herd membership, sex and age in African elephants in Chapters 5 and 6 (results for TGS, genital and buccal secretions, respectively), and the semiochemistry is characterised for adult females. Chapter 7 discusses all findings in context of the dissertations aims and results, and suggests avenues for future research.

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# Chapter 2: Scent discrimination of human odourtypes by African elephants*

*"Fascinating. Dogs can detect diabetes, cancer, and now Covid-19. If it's true that dogs are even more sensitive than PCR, needing fewer molecules, just imagine the implications, not just for medicine but for biology generally."*
*Richard Dawkins (2020)*

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**Abstract:**

This paper presents data on the success rate of African elephants in human scent matching to sample performance. Working with equipment and protocols similar to those used in the training of forensic canine units in Europe, scent samples were collected on cotton squares from twenty-six humans of differing ethnic groups, sexes and ages, and stored in glass jars. Three African elephants were trained to match human body scent to the corresponding sample. In total, four hundred and seventy trials, during which the elephant handlers were blind to the experiment details, were conducted. Each trial consisted of one scent that served as the starting (target) sample to which the elephant then systematically determined a potential match in any of the nine glass jars presented. Elephants matched target and sample at levels significantly higher than indicated by random chance, displayed no loss of working memory, and successfully discriminated target odours. They also discriminated between related human individuals spanning three generations and including sibling pairs. In addition to demonstrating scent matching capabilities, this experiment supported the elephants' significant ability to perform well at operant conditioning tasks.

**Key words**: African elephant, olfaction, scent discrimination, scent matching to sample

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## 2.1 Introduction

Elephants have the largest absolute brain in terrestrial mammals, with a large amount of cerebral cortex, especially in the temporal lobe, and a well-developed olfactory system. These structures are linked to complex learning and behavioral functions in humans, while elephant brain size itself may be associated with memory storage and intelligence (Shoshani et al. 2006). The enlarged neocortex is shared by other species such as humans, apes and certain dolphins; species that have been attributed with higher intelligence and cognitive abilities.

The elephant brain is considered a macrosmatic brain, since the rhinencephalon, or olfactory bulb, is very large. The enlarged rhinencephalon is associated with a keen sense of smell (Shoshani et al. 2006). An elephant's sense of smell may be five times as sensitive as that of a bloodhound, with neuroanatomical studies indicating a highly convoluted cerebral cortex and a series of 32 large olfactory turbinals, primary endoturbinals (Rasmussen et al. 2005) and an unusually extensive bilobed vomeronasal organ (VNO). Recent studies confirmed that elephants have an astonishingly large repertoire of olfactory receptor (OR) genes (Niimura et al. 2014). These ~ 2000 OR genes (humans have 400) may be linked to the elephant's heavy reliance on olfaction for fundamental life-strategy decisions during foraging, reproduction and social interaction.

Elephants also use their sense of smell to find food (Plotnik et al. 2014, Santiapillai & Read 2010), with olfactory cues in the physical environment influencing elephant behaviour and migration patterns (Douglas Hamilton 1975, Sukumar 2003). According to Rasmussen et al. (2000), chemical memories of landscapes, pathways, mineral sources, waterholes, coming rains or flooding rivers, and tree odours signifying seasons are key to elephant survival. When foraging near human settlement areas, elephants also use olfaction to detect and classify human threats into subcategories (Bates et al. 2007, McComb et al. 2014).

Dung and urine deposits on elephant pathways connecting core areas within home ranges (von Dürckheim 2011) are used to assess reproductive and fitness status of conspecifics (Sukumar 2003). Female elephants can detect when a conspecific is in oestrus from urine (Meyer et al. 2008), while males use the flehmen response in response to the oestrus

pheromone (urine or genitalia) and assess musth/non-musth status from temporal gland secretions and urine in other males. Studies have shown that elephants can detect pheromones in urine, breath and temporal glands (Rasmussen & Krishnamurthy 2000; Rasmussen 1988, 1995, 1998, Rasmussen & Schulte 1998).

Chemical signals and olfaction are also fundamental to the preservation of familial and non-familial relationships. Elephants live in complex fission-fusion societies, and the maintenance of social cohesion necessitates the ability to identify a large number of individuals. This is especially relevant as dominance hierarchies emerge along matrilineal lines when competition for resources is high (Wittemyer et al. 2007). Auditory evidence suggests that African elephants can recognize the contact calls of up to 100 individuals (McComb 2014), while olfactory research by Bates (2007) suggests that elephants keep track of their family members by monitoring chemical cues in urine. Elephant bond groups rejoin when conditions are favourable, and the greeting ceremony among African elephants is marked by intense urination, defecation, and temporal gland secretions. Although no empirical studies have explained the function of these chemical emissions, it is likely that these individual odour signatures underlie kin recognition in elephants (Wyatt 2003) and promote herd stability.

Experimental studies on habituated elephants allow for unique opportunities to further our understanding of the significance of olfaction to elephant behaviour: chemical communication in Asian elephants and the significance of musth and oestrus signals in particular were elucidated by extensive research into Asian elephants (Rasmussen 1988, 1995), with the subsequent identification and verification of a female-to-male sex pheromone ((Z)-7-dodecenyl-acetate) (Rasmussen et al. 1996a, Rasmussen et al. 2005). Evidence suggests that elephants display olfactory learning capabilities (Arviddson et al. 2012, Slotnick et al. 1991), have excellent long-term olfactory memory (Markowitz et al. 1975), and excel at olfactory discrimination tasks (Rizvanovic et al. 2013) among structurally related odourants. In this study, it was expected that African elephants are not only able to classify human ethnic groups into subgroups (Bates 2007), but are also able to discriminate between individual humans. Human beings have unique odour profiles which are stable and constant over time, which canines can recognize and use to discriminate among individuals (Curran, Rabin & Furton, 2005). The aim of this study was to test

olfactory discrimination in African elephants in a controlled field setting, and to collect data on olfactory discrimination performance using equipment and protocols similar to those used by the police working dog units in Europe (Schoon & De Bruin 1994, Marchal et al. 2016). The MTS procedure using a line-up addresses not only the elephants olfactory acuity, but their ability to respond correctly to operant conditioning and the systematic execution of tasks over a given time period.

## 2. 2 Methods:

### 2.2.1 Subjects

This research used three elephants (*L. africana*) from Adventures with Elephants (AWE) in BelaBela, South Africa (Table 1) to test MTS capability in African elephants. AWE controlled all aspects of husbandry, enrichment, handling and training. Three out of six elephants were selected to participate in the research as Shan, the other female was with calf, while Nuanedi (another mature female) would not separate easily from the mother and calf, and was therefore not selected for training in order to minimise stress. Elephants are housed together at night in 9m x 9m enclosures. The elephants are fed bana grass (*Pennisetum purpureum*), oat hay and lucerne (*Medicago sativa*) at night, and game pellets, cut branches and citrus during interactions with tourists. During the day, in between tourist interactions, elephants are let out into the 3000 ha reserve to roam and feed freely. The elephants are kept in a hands-on system, in which handlers have full access to the animals and they are therefore accustomed to follow commands. Elephant handlers have been in AWE's employ since 2008. Elephants are well habituated to people, and have been successfully utilised for scientific research on elephant morphology, scent detection, hormones and biochemistry, and communication infrasound communication (Baotic & Stoeger, 2017, Panagiotopolou et al. 2016, Miller et al. 2015, Zeppelzauer, Hensman & Stoeger, 2014, Stoeger et al. 2012). Training was conducted at the facility and disruptions to elephants' daily routine were minimised in order to limit stress (Stellenbosch University Ethics Approval Protocol Number: SU-ACUM15-00002).

**Table 1:** Age and sex related details of the three elephants used in human scent matching to sample trials.

| Elephant | Age | Sex |
|---|---|---|
| Mussina | 13 | Female |
| Chishuru | 18 | Male |
| Chova | 18 | Male |

### 2.2.2 Experimental Design

Elephants were trained to walk along a line-up of mounted glass jars in a free search, without direction from a handler riding the elephant, to smell the provided scent samples, and to alert if the target odour was encountered. Training procedures were adapted from Miller at al. (2015), where AWE elephants were trained to alert to a target scent by operant conditioning, and food was given as a reward for correct alerts. The line-up consisted of nine glass jars (80cm apart) in metal holders mounted on a 7m horizontal wooden pole at 1.6 m above the ground (Figure 1). Although up to seven jars are routinely used for training of highly specialised forensic canines in olfactory matching to sample tasks (Marchal et al. 2016, Pinc et al. 2011), we used nine jars for the MTS procedure given the elephant's superior olfactory and cognitive ability (Byrne & Bates, 2009). Glass jars had a diameter of 8.5cm, and were sealed with a metal lid, punctuated by holes. This allowed elephants to sniff the sample without soiling it. Between each session, glass jars were removed, rinsed with unscented soap and sundried to avoid any possible contamination. Elephants alerts differed markedly – Mussina alerted with a high foot salute and ears forward, Chova alerted by "freezing", with his trunk curled on the scent target and remaining immobile, while Chishuru alerted by bringing his ears forward and raising his head (Figure 2).

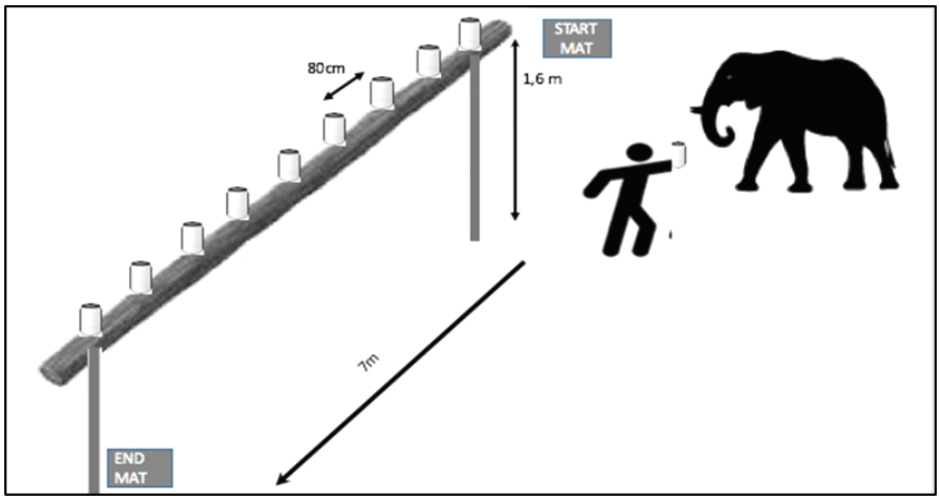


**Figure 1:** Experimental design of scent line-up using African elephants

a) (b) (c)

**Figure 2**: Alert behaviour of three African elephants during scent matching to sample performance Chova (a), Chishuru (b) and Mussina (c)

### 2.2.3 Odour Sample Preparation and Collection

For target samples, body odour from unspecified body parts was collected from 26 humans of four ethnic groups (Shona, Xhosa, Coloured and Caucasian), sexes and ages around the town of BelaBela. The scent donors consisted of 17 men, and 9 women, including two young children. Each individual wore four woven cotton fabric cloths (20 cm squares) on their body for a minimum of 15 minutes. Cloths were then sealed and stored in sterilized glass jars with removable metal caps. Cloths were used within two days of collection. All samples were handled by the same individual wearing latex gloves in order to avoid contamination, and cloths were indistinct from each other in colour. Cotton was used as it yields the greatest number of volatile compounds and the highest scent mass amount among textiles (Prada et al. 2011).

### 2.2.4 Training and testing procedure

#### 2.2.4.1 Training

Elephants were first trained to detect a single scent. During training, elephants were presented with a cloth containing the target scent, and the same cloth was then placed on top of a jar in the line-up in an established training area. As soon as the elephant regularly retrieved the target scented cloth on top of the jar, the target cloth was hidden in the jar covered by a metal screw-top lid with holes. Elephants were encouraged to place their trunk on the glass jar lid to smell the odour. The other eight glass jars remained empty. Elephants were asked to alert to the one jar holding the target scent. After elephants successfully completed the task, eight blank control cloths (Training 1) were added. This was followed by the introduction of three distractors (human odour not matching the target scent) and five blanks and the experiment repeated (Training 2). Visually sample types (target, distractor and blank) were indistinguishable from one another to ensure elephants and handlers could not make use of visual cues.

#### 2.2.4.2 Testing

Once elephants consistently retrieved the target scent, scent discrimination testing was conducted. Testing 1 and testing 2 (Table 2) were spaced three weeks apart due to AWE elephant availability, and both tests examined the ability of elephants to retrieve one target scent, with eight distractors, from the scent line-up. Testing 1 trials were run over 7 days, and testing 2 ran over 4 days. During both testing 1 and 2, the number of trials run on any day varied depending on elephant performance and stamina. Elephants were randomly provided with blank runs (all non-matching scents in entire line-up) throughout training and testing phases. Elephants that did not alert in the blank runs were rewarded at the end of the blank line-up with food and praise to reinforce “honest” responses. Target positions were randomised in xcel prior to testing, and only the observer and the assistant knew the location of the target sample. Throughout training and testing procedures, the elephant trainer, a handler, an assistant and an observer were present. The trainer and handlers roles were to 1) encourage elephants to sniff the ground 2) identify when an elephant was alerting to a target scent, 3) ascertain whether the alert was “honest” or “dishonest” and 4) confirm honest responses with the observer. The observer ensured independence of observations and recorded any changes

of elephant behaviour during the line-up, and kept the trainer and the handler blind to the location of the target scents during testing phases. The assistant replaced used glass jars between trials with uncontaminated glass jars after each completed line-up out of sight of the trainer, handler and elephant.

#### 2.2.5 Response Accuracy

For testing, correct and incorrect response frequencies were assessed against chance predictions using Chi Square. A Two Way Anova and Levene's Test for Homogeneity of Variances was used to test whether a decline in performance could be detected from the beginning towards the end of the scent line-up. Microsoft Excel was used for the Chi-square calculations, and Statistica V13 (Dell Inc. (2015)) for the 2-way ANOVA. Results were considered statistically significant at $p < 0.05$.

## 2.3. Results

### 2.3.1 Matching to sample performance

In total, elephants indicated the target scent correctly 387 times (82%) out of the 470 trials performed (81% for Chova, 86% for Mussina and 73% for Chishuru). Mussina the female was the slightly more successful at scent-matching to sample performance than the males with a mean percentage of correct responses of 88.35%.

#### 2.3.1.1 Training

During training 1, elephants were exposed to 1 target human scent and 8 blanks: One hundred and eleven training trials were conducted, during which the target scent was correctly indicated 101 times (91%). For Training 2, elephants were exposed to 1 target, 3 distractors and 5 blanks, with a combined total of 31 trials. The target scent was detected 25 times (81%).

#### 2.3.1.2 Testing

During testing 1 and 2, elephants were exposed to 1 target scent and 8 distractor odours. During testing 1, 129 trials were conducted and the correct target identified 111 times (86%). During testing 2, elephants were exposed to 199 trials, and correctly identified the target scent 150 times (75%).

**Table 2:** Results of Chi Square analyses on both correct and incorrect responses made by three African elephants on human scent matching to sample training and test phases. Significant results* indicate that indication frequency differed from those predicted by chance.

| Type of Line up | No of distractors | Elephant | No correct responses | No line-ups | Chi-square p-value | % correct |
|---|---|---|---|---|---|---|
| **Training 1** | | | | | | |
| | 1 target 8 blanks | Chova | 33 | 33 | <0.01* | 100.00% |
| | 1 target 8 blanks | Mussina | 36 | 42 | <0.01* | 85.71% |
| | 1 target 8 blanks | Chishuru | 32 | 36 | <0.01* | 88.89% |
| **Training 2** | | | | | | |
| | 1 target 3 distractors, 4 blanks | Chova | 8 | 11 | <0.01* | 72.73% |
| | 1 target 3 distractors, 4 blanks | Mussina | 8 | 9 | <0.01* | 88.89% |
| | 1 target 3 distractors, 4 blanks | Chishuru | 9 | 11 | <0.01* | 81.82% |
| **Testing 1** | | | | | | |
| | 1 target, 8 distractors | Chova | 29 | 37 | <0.01* | 78.38% |
| | 1 target, 8 distractors | Mussina | 47 | 55 | <0.01* | 85.45% |
| | 1 target, 8 distractors | Chishuru | 35 | 37 | <0.01* | 94.59% |
| **Testing 2** | | | | | | |
| | 1 target, 8 distractors | Chova | 50 | 67 | <0.01* | 74.63% |
| | 1 target, 8 distractors | Mussina | 57 | 66 | <0.01* | 86.36% |
| | 1 target, 8 distractors | Chishuru | 43 | 66 | <0.01* | 65.15% |

### 2.3.2 Working memory

For all elephants, correct and incorrect responses were calculated for target sample per position in the scent line-up (position 1 to 9). Positions were categorised into close (position 1-3), near (position 4-6), and far (position 7-9) as declines in performance towards the end of forensic scent line-ups have been recorded in working dogs (Horowitz 2014). Cumulatively, the elephants responded correctly with a mean of above 80% per category, and elephant accuracy did not decline towards the end of the line-up with performance differences between categories highly insignificant at p =0.829, 0.95 Confidence Interval (Figure 3). As pertaining to learning/ceiling effects, male performance over the three category positions (close, near, far) was more variable than the female but not significantly so (Figure 4). It would seem as if the female was generally more consistent than the two males. However, to test whether there was in fact a ceiling/learning effect, more data from more animals (of both sexes) would need to be evaluated over more time points.

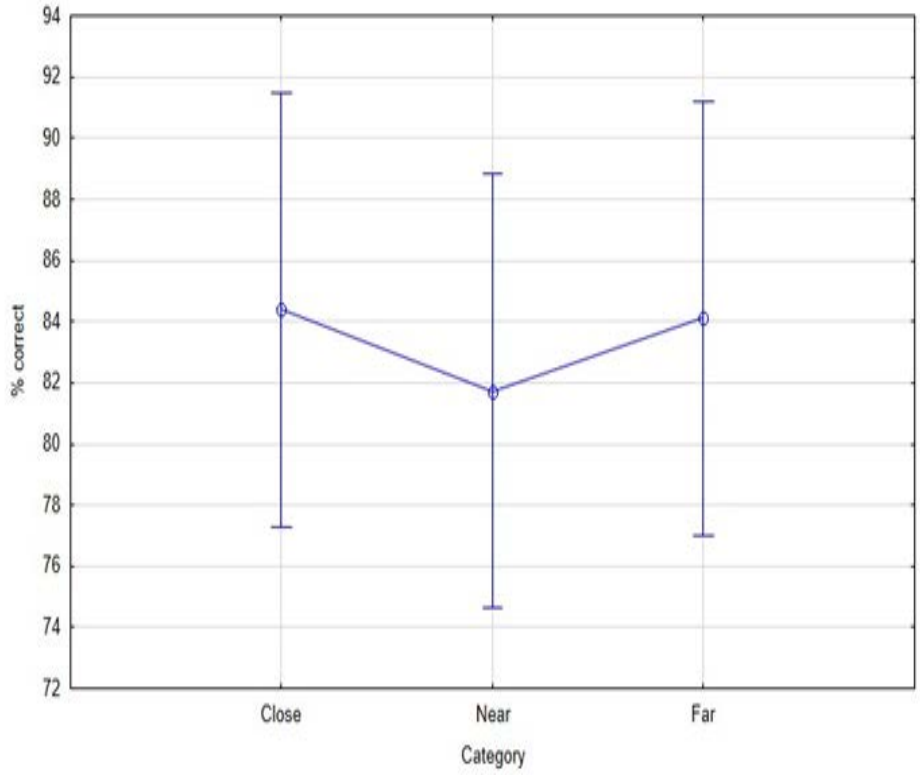


**Figure 3:** Mean % correct response of elephants per target odour position

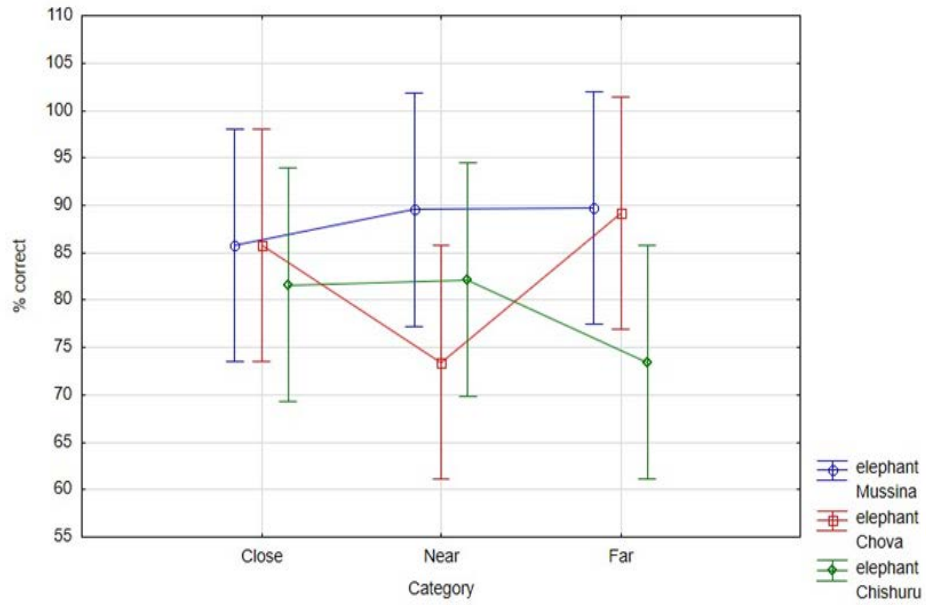


**Figure 4:** % correct response of individual elephants per target odour position

### 2. 3.3 Scent discrimination among related individuals

Interestingly, elephants were able to discriminate between closely related humans that included grandparents, parents, offspring, and sibling odours. In 46 of the trials, elephants were exposed to between 2 and 4 related humans in any one 9 scent line-up. Elephants were successful 93% of the time in identifying the correct target odour (Table 3).

**Table 3:** Number of trials containing odour from related individuals.

| Elephant | Trials with odour from closely related humans | No of correct responses | % correct |
|---|---|---|---|
| Chova | 15 | 13 | 86.67 |
| Mussina | 20 | 20 | 100.00 |
| Chishuru | 11 | 10 | 90.91 |
| Total | 46 | 43 | 93.48 |

## 2.4 Discussion

Our results demonstrate that African elephants can be trained to efficiently match a target human scent to a matching sample using canine training protocols. The results reflect previous research that elephants display olfactory learning ability (speed of initial task acquisition and intramodal stimulus transfer) which is at least as good as that of mice, rats, and dogs, and superior to nonhuman primates and fur seals (Arvidsson et al. 2011). Evidence supporting generalized Matching To Sample (MTS) has been recorded for several non-human species, including rats (Pena et al. 2006), primates (Barros et al. 2002), sea lions (Pack et al. 1991, Kastak & Schusterman 1994), and dolphins (Herman & Gordon, 1974). Matching to Sample using olfactory stimuli has been comprehensively tested in dogs (Canis familiaris), with dogs able to successfully scent-match species such as maned wolves (Chrysocyon brachyurus) (Wasser et al. 2009), Amur tigers (Panthera tigris altaica) (Kerley & Salkina, 2007), and humans (Kalmus 1955; Settle et al. 1994, Schoon & De Bruin 1994, Schoon 1996, Jezierski et al. 2010). The elephants could discriminate between nine different human scents in a nine scent line-up, and between twenty-six different human scents in any one day. Anecdotal evidence suggests that elephants are able to recognize individual humans (Ritchie 2009), while empirical research into mammal recognition of individual humans has been found in chimpanzees (Boysen and Berntson, 1986), pigs (Tanida et al. 1995) sea lions (Schusterman et al. 1992), cattle (Taylor & Davis, 1998) and dogs (Settle et al. 1994). Olfactory discrimination and recognition in elephants are fundamental to social cohesion, communication, kin discrimination, individual recognition, dominance and mate choice, it is thus unsurprising that elephants can discriminate between humans (Bates et al. 2008, Greenwood et al. 2005, Bagley et al. 2006).

The elephants further successfully distinguished between the odour of four related humans (siblings of similar ages, parents and grandparents), where one scent was the target and the other three were used as distractor odours in a nine-scent line up. Humans have individual odourtypes as a result of their genotype, which regulates metabolic processes which result in unique body odours. Specifically, odour associated with the Major Histocompatability Complex (MHC) is suggested to play a significant role in individual and kin recognition as well as reproductive behaviour in humans (Wedekind et al. 1995), mammals (Penn 2002, Brown &

Eklund, 1994) including possibly elephants (Archie et al. 2010). Dogs can successfully discriminate body odours of related humans (Harvey et al. 2006) and highly certified forensic dogs can be efficiently trained to reliably differentiate even monozygotic twins (Pinc et al. 2011). Volatile Organic Compounds (VOC) from human skin can function as a biometric marker and diagnostic tool because an individual's odour print is sufficiently unique (Curran et al. 2005, 2010). Human skin VOCs contain ketones, aldehydes and amines (Prada et al. 2011, Gallagher et al. 2008), compounds which Asian elephants could detect in an olfactory discrimination test of structurally related odourants (Rizvanovic et al. 2013).

In forensic science, human scent is increasingly being examined as a source of trace evidence for investigative purposes in law enforcement applications, specifically in the realm of canine detection tools. Forensic canines have been employed in Search and Rescue (SAR) operations, human tracking and as a biosensor tool in detecting explosives (Goeth et al. 2003, Furton & Myers 2001), narcotics and human remains due to their high sensitivity and selectivity towards certain odours (Furton et al. 2015). In fact, recent research into biosensor detection revealed that African elephants could accurately and reliably detect an explosive odour (Miller et al. 2015), with specificity and sensitivity scores of over 90% as stipulated by the Scientific Working Group for Dog and Orthogonal Detector Guidelines (SWGDOG).

The elephants in this study also displayed no decline in working memory. In scented line-ups, studies on expert detection dogs have shown a decrease in working memory by as much as 40% (Horowitz 2014, Porrit et al. 2015), with dogs having less of a 15% chance of making an accurate match towards the end of the six scent line-up, despite the fact that they were working with essential oils, which do not degrade easily. In studies on elephant cognition and memory, elephants were capable of remembering the reward value of previously learned odour stimuli after 2, 4, 8, and 16 weeks of recess without any signs of forgetting (Arvidsson et al. 2012), while Rasmussen (1995) found evidence of filial-maternal recognition through urinary signals after 2-27 years of separation. Rizvanoviz et al. (2013) suggests that this olfactory discriminatory ability is correlated not with rhinencephalon size but with the number of olfactory receptor (OR) genes. Elephants have the highest number of OR genes recorded in any mammal - 2000 OR genes compared to canines' 800 OR genes (Niimura et al. 2014).

The discriminatory power of our habituated elephants is highly advantageous in forensic applications due to the capability to be rapidly trained on multiple odours with a high degree of specificity. To use it in a law enforcement context, South African forensic experts could collect scents from the scene of a crime. This odour sample could be an object, such as an item of clothing or mobile phone, or cotton pads strategically placed to capture scent left behind at scene of a crime. These odour samples can be stored in sterile conditions for long periods of time. The police then collect an odour sample from the suspect or victim, as well as from other individuals (distractor odours) not related to the case, and place them in jars. The elephant can then perform a scent-line up, sniffing the sample “evidence” scent collected at the scene, and comparing it to odours in the line-up. When the odours match, the elephant is trained to indicate the target sample.

However reliable training procedures and testing protocols would have to be developed with South African legal and forensic experts for the method to gain admissibility and validity in a court of law. In South Africa for example evidence from a highly trained Belgian Malinois led to the conviction of two rhino poachers in the Kruger National Park in a historical landmark ruling (Times Live, 2015). Central to the admissibility of evidence in court was this dog’s superior scenting training and capability, as inter-individual differences in olfactory ability, memorization of odours, performance at operant conditioning and motivation to sniff all scents in a line-up may influence the training process in animals (Jezierski et al. 2010). Inter-individual differences were also noted in our elephants, with Mussina, the female, performing consistently and keenly across all training and testing phases (Table 2). High olfactory acuity in female elephants would not be surprising, given the fact that female elephants remember the contact calls of extended family of up to 100 individual elephants, and use exudates in urine to monitor the location of at least 30 family members (Bates et al. 2007). In fact, sexual dimorphism in the olfactory bulb was found in humans, with women having more neurons and glial cells in the olfactory region than men (Pinto et al. 2014). The possibility of heightened olfactory sensitivity in female elephants warrants further investigation.

## 2.5 Conclusion

In this study, elephants displayed the capability of performing well at operant conditioning tasks, accurately matching human scents to sample, with no loss of working memory. This demonstrated the elephants' olfactory ability to not only contextualise and classify humans in categories, but to accurately discriminate between individual human scents, also among related individuals. Although only three elephants were tested, we have no reason to believe these results are not representative of *L. africana.*

The ability of habituated elephants to meaningfully contribute in future to forensic odourology, disease detection and wildlife surveys en par with canines (Smith et al. 2003, Willis et al. 2004, Cabik & Heaton, 2006) shall, due to the pachyderm's superior cognition, close familial ties, conservation status and public reverence, remain a contested topic. Although this study has shown that elephants are capable of matching to sample in a forensic line-up, further application should be critically evaluated and ethically defensible.

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**Conflict of interest statement:**

All authors declare that there are no conflicts of interest.

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# Chapter 3: Odour mediated navigation and scent trail tracking ability in African elephants

*"The nose, for example, of which no philosopher has ever spoken with veneration and gratitude – the nose is, albeit provisionally, the most delicate instrument at our disposal. It is an instrument capable of recording the most minimal changes that escape even spectroscopic detection."*

*Friedrich Nietzsche (1889)*

**Abstract:**

The ability of elephants to track or follow scent trails is fundamental to their ethology: it enables them to navigate their territory, avoid danger, find food and identify offspring or mates, requiring only faint olfactory molecules to do so. Elephants are primarily olfactory animals as evidenced by their well-developed olfactory system, large olfactory bulb, and impressively high number of olfactory receptor genes. While there has been growing interest in the olfactory and cognitive ability of African and Asian elephants, scent trail tracking has not been studied in a controlled environment. This study showed that African elephants can track human scent trails. We created a behavioural task whereby three elephants were trained to navigate a human scent trail to an odour source paired with a food reward. Elephants navigated the scent trails in an open environment using naturally fluctuating ground borne odour cues as elephant positions were recorded in real time. Results showed that i) elephants can accurately scent track a human odour trail and ii) elephants are able to discriminate between two types of scent trails – the original trail, and a distractor trail laid half an hour later by a different person. To generate odour-mediated orientation, elephants were trained using operant conditioning procedures and trail laying methodologies adapted from forensic protocols for military sniffer dogs (SWGDOG). This is the first study to demonstrate that African elephants can successfully discriminate between and track human scent trails. African elephants also secrete chemicals from their toes, and this research begs the questions whether elephants use chemical compounds from these secretions on elephant pathways to track oestrus/musth individuals in this spatially dispersed species. And improved understanding of elephant tracking behaviour could contribute positively towards habitat preservation and resettlement success by manipulation of release and holding sites of conspecifics.

**Key words:** African elephant, cognition, olfaction, scent tracking, scent trails

## 3.1 Introduction

Olfaction is central to vertebrate survival and reproduction. The two central roles of olfaction are discriminating between odourants and pheromones with diverse chemical structures and features, and to spatially navigate their source (Jacobs 2012). Animals respond to scent by either avoiding the signaller (predators or dominant competitors) or pursuing the scent to the signalling source (e.g. receptive conspecifics or food). The ability of animals to track odour trails is critical to their survival; it enables animals to navigate their home range, source food, identify offspring or mates and avoid predators, requiring only faint odour cues to do so. Even in the aquatic environment, mammals such as the star-nosed vole (*Condylura cristata*) and planktonic shrimp use chemosensory tracking of underwater scent trails to find prey/nutrients (Catania 2006, Hamner & Hamner 1977). Research into odour tracking behaviour and ability in land mammals is limited to mice, rats, dogs and humans (Jones & Urban 2018, Khan et al. 2012, Wallace te al 2002, Hepper & Wells 2005, Porter et al 2007), while the scientific literature on elephant tracking ability is non-existent.

Elephants are a remarkable animal model for addressing olfactory questions as they are macrosmatic, have an extraordinary sense of smell and exhibit significant cognitive ability. The elephant brain is comparable in size and complexity to those of cetaceans and humans (Hakeem et al. 2005). Elephant's olfactory acuity – their ability to detect odours at low concentrations – is excellent and they can learn to recognize various odours, and have the ability to detect and discriminate between odours (Miller 2015, von Dürckheim et al. 2018). Elephants rely heavily on chemosensory signals for various behaviours, such as conspecific interactions, migration, reproduction, foraging and predator avoidance (Plotnik et al. 2014, Bagley et al. 2006, Bates et al. 2007). Elephants have numerous morphological adaptations to support significant olfactory acuity - a large olfactory bulb, with the highest number of functional olfactory receptor genes in mammals. Olfactory receptors (ORs) detect odours in the environment, and OR genes constitute the largest multigene family in mammals (Niimura et al. 2014).

The aim of this study was twofold: 1) to assess whether African elephants are able to track a scent trail using ground borne olfactory cues, and 2) to assess whether African elephants can discriminate between different human scent trails.

## 3.2 Methods

### 3.2.1 Subjects

This research used three elephants (*L. africana*) from Adventures with Elephants (AWE) in Bela-Bela, South Africa, to test scent-trail tracking capability in African elephants. AWE controlled all aspects of husbandry, enrichment, handling and training. Three out of six elephants were selected (Table 3.1) to participate in the research as the two females, Shan and Mussina, would not separate easily from their calves, and were therefore not selected for training in order to minimise stress. Elephants are housed together at night in 9m x 9m enclosures. The elephants are fed bana grass (*Pennisetum purpureum*), oat hay and lucerne (*Medicago sativa*) at night, and game pellets, cut branches and citrus during interactions with tourists. During the day, in between tourist interactions, elephants are released into the 3000 ha reserve to roam and feed freely. The elephants are kept in a hands-on system, in which handlers have full access to the animals and they are accustomed to follow commands. Elephant handlers have been in AWE's employ since 2008. Elephants are well habituated to people, and have been successfully utilised for scientific research on elephant morphology, scent detection, hormones and biochemistry, laterality, olfaction, Social Network Analysis, and infrasound communication (von Dürckheim et al. 2018, Panagiotopolou et al. 2016, Miller et al. 2015, Zeppelzauer, Hensman and Stoeger, 2014, Stoeger et al. 2012). Training was conducted at the facility and disruptions to elephants' daily routine were minimised in order to limit stress (Stellenbosch University Ethics Approval Protocol Number: SU-ACUM15-00002).

**Table 3.1:** Age and sex related details of the three elephants used in odour tracking tests.

| Elephant | Age | Sex |
|---|---|---|
| Nuanedi | ~18 | Female |
| Chishuru | ~22 | Male |
| Chova | ~24 | Male |

### 3 2.2 Experimental Design

The three elephants were trained to track human scent trails in the direction the trail layer had moved. Elephants respond well to operant conditioning tasks and a food reward system (oranges) was used to teach the elephants to ground track the human scent trail, and to reward the elephant at the successful completion of each scent-trail tracking test. Odour perception can be difficult if tracking motivation is low (Neureither et al. 2017). Odour has been shown to mediate foraging activities indirectly by providing odour trails or signs related to optimal feeding sites. Incomplete or unsuccessful scent trail tracking were not rewarded. The experiments were not designed to test the elephants' ability to assess trail direction or assess tracking behaviour. Rather, we were interested whether elephants could follow the scent trail by ground-tracking. During training and testing, all elephants were tracking in a hands-on system, with handlers blind to the direction of the track.

### 3.2.3 Training and testing procedure

#### 3.2.3.1 Pre-screening

Initially, five elephants were tested, including two females and their calves. Scent trails were laid by leaving a food stimulus associated to the food reward (oranges) to mark the start of the human scent trail. Food items were placed every meter along the trail with wind blowing from directly behind the track layer so that the elephant had to follow the trail to find the reward. Elephants were trained to ground track and sniff the odour plumes above the ground only, rather than using air-borne odour plumes, as the study aimed to evaluate whether elephants could track humans to the end of a human scent trail, even after the person may have left the scene. The purpose of the easy track was to determine which elephant displayed characteristics suitable for the tracking. It was determined that Chova, Nuanedi and Chishuru would be suitable candidates.

#### 3.2.3.2 Training

Once selection of tracker elephants was completed, simple, straight line tracks 100 meters in length were laid (Hepper & Wells, 2005). Ground tracking, rather than air scenting or trailing, is described in canine forensic literature as "following the trail with head down and noses on path, very closely following the footsteps of the individual (Hepper & Wells, 2005)." Elephants were

encouraged to navigate towards the end of the track using the human trail as the olfactory cue leading to the food reward site, where they were given the oranges upon successful completion of the track. Regular food rewards were provided in order to reinforce tracking behaviour (Khan et al. 2012). The elephants all tracked very well and followed the laid trails with good fidelity. Once elephants comfortably tracked the 100m scent trail, gentle turns were introduced and trail length extended to 160m, with food rewards placed further apart and tracks allowed to age for 10 minutes before the elephant began tracking. The elephant continued following the track with high fidelity. The aim of the training was to gradually increase track complexity (length, age and number of turns, and frequency of rewards) to meet assessment criteria, adapted from Florida International University's "Scientific Working Group on Dog and Orthogonal detector Guidelines" (SWGDOG).

### 3.2.4 Testing

Once elephants consistently tracked the scent trail, testing was conducted. Twenty-nine 200m scent trails ("poacher" trails) were laid by the trail layer in random directions over multiple surfaces one hour before testing. Trail and distractor layers were selected randomly. The distractor scent trail was laid by a second person thirty minutes before the start of the test. The distractor cut across the trail at 90 degrees (Figure 3.1) as per SWGDOG specifications. Food rewards were eliminated from the trail. The end of the trail was marked by a pile of oranges (the food reward). The elephant had 30 minutes to complete the test, after which the test was cancelled. The elephant and handler were not present during the scent trail laying. The scent trail began with the elephant led to the start of the trail by the trainer, which was marked by a scuff-mark from the trail layer's boot. Previous research suggests that trained animals conduct a chemosensory analysis of a target odour that, by being coupled with operant conditioning and food rewards, is assigned subjective hedonic valence (Gadziola et al. 2015) and memorised as an olfactory object (Neureither et al. 2017). This type of sensory learning aids the cognitive processes required in promoting perceptual stability of an odour, by increasing tracking motivation. The elephant was encouraged to sniff the ground, after which the order to "search" was given by the trainer. The original trail, the distractor trail and the actual search path were

recorded with a Garmin GPS and imported into ArcGIS. The distance between the elephant's body position and the original trail was used as a measure of tracking accuracy. For each tracking test wind direction, humidity, surface types and time to completion were recorded. Testing was run over 12 consecutive days. The number of trials run on any day varied depending on elephant availability. Each scent trail was only used once.

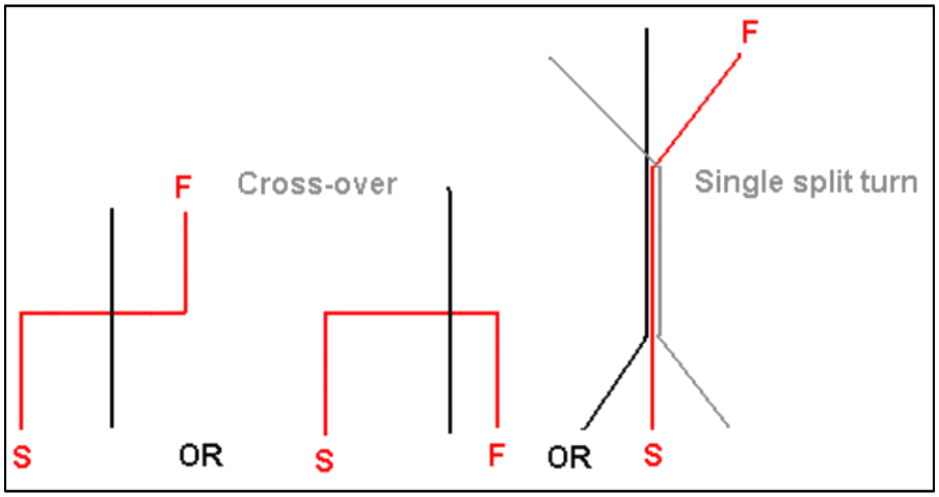


**Figure 3.1:** The cross-over track or trail designs used in elephant testing adapted from SWGDOG. S is the starting point for the human target, F is finishing point. In a cross-over design, the target makes two turns and his track or trail is crossed once by a distractor fresher track or trail.

### 3.2.5 Statistics

Repeated Measure Correlation was used to characterize the relationship between multiple environmental variables for each scent trail (R package 'rmcorr' version 0.4.1). Repeated measures correlation (*rmcorr)* is a statistical technique for determining the common within-individual association for paired measures assessed on two or more occasions for multiple individuals.

### 3.2.6 ArcGIS

Arcmap 10.6.1 was used for calculations and the Euclidean Distance tool used on each trail layer's points, creating a raster with distances around the points. The "Extract Multi Values to Points" tool was utilized to determine the distance of the tracking elephant to the nearest trail layer point. The "Extract Multi Values to Points" tool, extracts cell values at locations specified in a point feature class from one or more rasters, and records the values to the attribute table of the point feature class.

## 3. 3 Results

Table 3.2 presents the summary data from the testing phase in which elephants followed paths generated by human movement across the open landscape. Twenty-nine trails were laid, with an average distance of 185m. On average, 173 GPS coordinates were recorded per trail. Maximum trail age was 89 mins.

**Table 3.2:** Summary data of scent trail tracking results

| **Summary data** | |
|---|---|
| No of trails | 29 |
| Average No of GPS fixes/trail | 173 |
| Average length of trail | 185m |
| Average age of trail | 61 minutes |
| Minimum age of trail | 52 minutes |
| Maximum age of trail | 89 minutes |
| Average distance tracker to trail | 4 metres |
| Minimum distance tracker to trail | 2 metres |
| Maximum distance tracker to trail | 17 metres |
| Average time/track | 5 minutes |
| Maximum time/track | 10 minutes |
| Minimum time/track | 3 minutes |

Elephants were able to follow the scent trail in the correct direction of the trails more accurately than one would have expected by random chance. In total, elephants successfully completed the track 28 times (96%) out of the 29 trials performed. Chishuru was only used twice as his readiness to consistently track the trail waned, and therefore precluded him from further tests. Individually, Chova and Nuanedi achieved a significant level of performance. Elephants successfully discriminated between the two trail types, effectively ignoring the human distractor trail 100% of the time. It was not the aim of this study to analyse the tracking behaviour of the elephants during tracking, however it was noted that elephants extended their trunks to right above the ground, with the head held low. Elephants oscillated their heads from left to right, with trunks sweeping an 180 degree arc in front of the front feet, presumably when the scent signal became faint (Figure 3.2).

**Figure 3.2:** While following a human scent trail the head was held low and the trunk was extended above the ground (left), with oscillating head/trunk movements across the trail during tracking (middle and right).

Table 3.3 presents the *rmcorr* values for all sets of environmental variables tested. Although none of the environmental variables significantly impacted elephant tracking effectiveness, with elephants performing well across temperature, wind, humidity and surface gradients, both temperature ($p < 0.07$) and humidity ($p<0.07$) tended towards significance, suggesting that these two factors may play a role in tracking speed. Indeed, both tracking speed (duration and duration/m2) and accuracy (distance) had a negative rmcorrr relationship with temperature (-0.35), suggesting that elephants tracked faster at higher temperatures, while the positive rm correlation between humidity and duration (0.35) indicates that elephants took longer to track when humidity levels were high or when rain had fallen after the track had been laid.

In particular, on 7 December, both Chova and Nuanedi struggled significantly with tracking (Figure 3.3) after rain had fallen on to the trail prior to and during the tracking event, with GPS fixes indicating Nuanedi's circular searching behaviour off the track to locate the path, using olfaction to guide path integration (Etienne & Jeffrey 2004), and returning to the original track.

**Table 3.3:** Environmental variables tested against speed of track (duration) and accuracy of track (average distance) in 29 tracking trials.

| Variable 1 | Variable 2 | rmcorr | p value | lower 95% | upper 95% |
|---|---|---|---|---|---|
| Wind Speed | Duration | 0.1 | 0.64 | -0.31 | 0.47 |
| Wind Speed | Average Distance | 0.12 | 0.56 | -0.29 | 0.49 |
| Wind Speed | duration/m | 0.1 | 0.64 | -0.31 | 0.47 |
| Humidity | Duration | 0.35 | 0.07* | -0.05 | 0.66 |
| Humidity | Average Distance | 0.28 | 0.16 | -0.13 | 0.61 |
| Humidity | duration/m | 0.35 | 0.07* | -0.05 | 0.66 |
| Temp | Duration | -0.35 | 0.07* | -0.66 | 0.05 |
| Temp | Average Distance | -0.18 | 0.38 | -0.53 | 0.23 |
| Temp | duration/m | -0.35 | 0.07* | -0.66 | 0.05 |

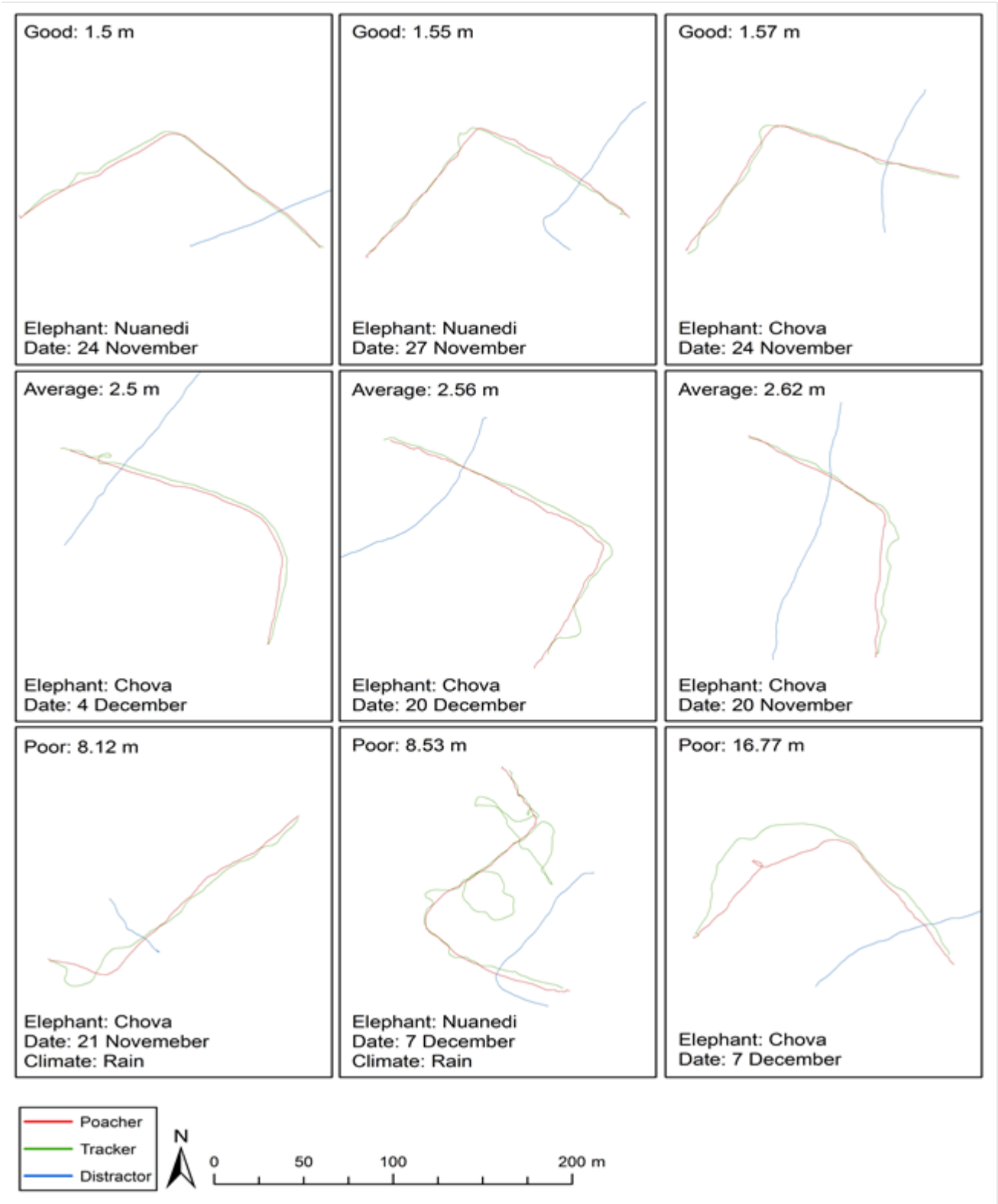


**Figure 3.3:** Elephant tracking performance – in *good* track performance (top), the elephant trunk was <1.5 m from the original trail. In average track performance (middle), the trunk was < 3m from original trail, and in poor track performance (bottom), the trunk was >8m from the original trail. The original trail ('poacher") is shown in red, the distractor trail laid thirty minutes later is shown in blue, and the path of the tracking elephant taken one hour after the original trail was laid is shown in yellow.

## 3.4 Discussion

These results demonstrate that African elephants 1) have the ability to track a scent trail using ground tracking, and 2) can successfully discriminate between different types of scent trails. Additionally, environmental conditions did not significantly impact tracking effectiveness. These findings provide novel evidence that elephants are highly adept at using ground born odours to navigate an odour source by actively sampling surface-borne odours across a variety of temperatures, humidity levels, ground surfaces and ground absorption types. This performance is in agreement with studies on other species (with fewer olfactory receptors). Dogs (*Canis lupus familiaris*) (Beebe et al. 2016, Thesen 1993, Hepper & Wells 2005), rats (*Rattus norvegicus*) (Khan et al. 2012, Wallace et al. 2002), humans (Porter et al. 2007), moths (*Heliothis spp*) (Vickers et al. 2006), and ants (*Linepithema humile*) (Perna et al. 2012) have shown the ability to track intra-and interspecies odour trails. Tracking ability is not only restricted to terrestrial animals however, with the semi-aquatic star-nosed mole (*Condylura cristata*) and water shrew (*Sorex palustris*) exhaling and re-inspiring air bubbles for underwater sniffing of scent trails and objects (Catania 2006). This odour tracking behaviour was witnessed in African elephants, when conspecific urine patches on the ground were investigated. Elephants blew air from the trunk, presumably to stimulate an air bubble of volatile scent molecules, which were then re-inhaled (personal observation).

Elephants have highly developed olfactory and vomeronasal systems suggesting that olfaction plays a major role in behavioural responses and decision-making (Niimura et al. 2014). Studies have shown that elephants use olfactory cues in the environment to navigate towards a food source, are able to discriminate between human beings using scent, distinguish between conspecific scent (Plotnik et al 2014, Bates et al. 2007). At a very fine scale, elephants can discriminate between chemical odour pairs differing in only one carbon chain length, with their olfactory acuity assumed to be as good as that of mice and superior to that of humans and nonhuman primates (Rizvanovic et al. 2013).

Gadbois and Reeve (2014) suggest that there are three processes to localize olfactory stimuli: searching, trailing and tracking. Elephants quickly learnt that the best searching strategy was to track the human scent trail to find the food reward. In species with only one olfactory

sensor such as the elephant, animals rely on self-movement and comparisons of olfactory concentrations over time in order to track the local odour gradient. In this experiment, elephants oscillated their heads across the scent trail in response to weakening odour signals. Rats too use head oscillations that sweep back and forth across a trail as they track it (Khan et al. 2012), a strategy also applied by humans (Porter et al. 2007) and ants (Draft et al. 2018, Hangartner, 1969). Dogs, insects (Vickers 2000), crustaceans, fish (DeBose & Nevitt, 2008) and cockroaches use a zigzagging approach to follow an odour trail.

Elephants successfully ignored the olfactory cues in the distractor trail 100% of the time. This is in agreement with Wallace (2002) who showed that rats could discriminate between different types of odour trails. The above average performance of the tracking elephants poses the question on how the animals are able to distinguish the target odour from a complex chemical background. The nature of the odour plume around each human foot print in each of the two scent trails implies that the plume contains two basic sources of information: 1) the chemical content of the signal and 2) the distribution of odourous molecules in time and space. It is not clear which chemical content in the plume the elephants detected - the scent of crushed vegetation, scent of shoe polish, scent of person-specific human body odour (VOCs), the scent of the soil itself that is triggered by pressure of a footprint, or a complex signature mix combining all of these. A study by Buzuleciu et al. (2016) indicated that scent associated with disinterred soil is the primary olfactory cue used by raccoons to identify locations of diamond-backed terrapin (*Malaclemys terrapin*) nests. Previous studies suggest that elephants can detect chemicals at very low concentrations, including those that constitute human odour types (von Dürckheim et al. 2018). If just 1/1000ml of human sweat (humans produce 800 $cm^3$ of perspiration in 24 hours) penetrates a human shoe, each footprint would contains 2.5 x 1011 molecules of butyric acid (Jurczyk-Romanowska, 2010). This would be over a million times above a dog's scent threshold concentration (and higher for elephants) and could be detectable in 28$m^3$ of air. Human skin is rich in olfactory cues – volatile organic compounds (VOCs) contain ketones, aldehydes and amines (Prada et al. 2011, Gallagher et al. 2008), compounds which Asian elephants could detect in an olfactory discrimination test of structurally related odourants (Rizvanovic et al. 2013). Studies have shown that elephants can discriminate between the body

odours of at least nine humans, including related individuals (von Dürckheim et al. 2018) and are able to detect and sub-classify African pastoralists by scent and by sound (Bates et al. 2007, McComb et al. 2014).

Lastly, these results further demonstrate that the elephant scent trail tracking was successful under a range of experimental conditions. Temperature, wind speed, barometric pressure and humidity influence the quality and intensity of odour in space and time (Conover 2007), yet elephants could track the human scent trail across varying wind speeds, wind directions, humidity and temperature gradients. The model selection process suggested that both humidity and temperature were correlated with the time it took to complete the trail, although the effects of these factors were equivocal. Trails were laid across varying humidity gradients. One would expect that higher humidity in Bela-Bela's climate (mean relative humidity during trials was 43.51%, min =20.2%, max = 87.7%) would assist bacterial decomposition of scent rafts, presumably producing odours that elephants can detect (Syrotuck 1972). Humidity may increase the efficiency of dogs in tracking and searching tasks by increasing odour intensity (Jenkins et al. 2018), but high humidity coupled with rainfall had the opposite result, with elephants struggling to stay on the scent trail. Hard rainfall and hot sunny days negatively impact depositional odour trail persistence and tracking ability in predators (Conover 2007). The mechanism behind the inverse relationship between temperature and elephant tracking speed is more difficult to explain. Studies seem to suggest that depositional odour trails can disappear in under 15 minutes in high temperatures of above 30◦C, while in overcast conditions and in thick vegetation, trails may last up to 48 hours (Conover 2007, Gutzwiller 1990). Research on odour navigation in rodents has focused on localization of airborne odours (Catania 2013, Bhattacharyya & Bhalla 2015; Gire et al. 2016), where a consistent wind direction would play an important role for source localisation. However, air scenting ability was not tested in this study as the aim was to establish whether elephants could track a human scent trail in order to localise a rewarding food source and the former mentioned might be an interesting aspect for future research. Future olfactory research should evaluate aerial sniffing behaviour, olfactory detection threshold (ODT) and odour mediated search strategies in elephants for inter-specific species comparisons Species with far fewer olfactory receptors, and smaller olfactory /cognitive

features have been shown to be able to detect a variety of chemical compounds (rats detect compounds between 0.04-0.10ppt) and spider monkeys at 1 ppt (Wackermannová et al. 2016).

## 3.5 Conclusion

The results confirm that elephants were proficient at following and discriminating odour trails, successfully using olfactory cues of a human trail to navigate towards a desirable food source. The results provide an essential link between olfaction, spatial behaviour and the higher-order neural processes that enable an elephant to distinguish between types of odour trails and the importance of these trails. Behavioural relevance of olfactory ability in elephants would allow researchers to better appreciate chemosignalling with regards to kin and individual recognition, communication, food choice, and resource use. African elephants also secrete chemicals from their toes, and this research begs the questions whether elephants use chemical compounds from these secretions on elephant pathways to track oestrus/musth individuals in this spatially dispersed species. And improved understanding of elephant tracking behaviour could contribute positively towards habitat preservation and resettlement success by manipulation of release and holding sites of conspecifics.

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# Chapter 4: Evidence of individual odour and individual recognition in African elephants

*"In most cases, when during the breeding-season the male alone emits a strong odour, this probably serves to excite or allure the female … The odour emitted must be of considerable importance to the male, inasmuch as large and complex glands…have in some cases been developed. The development of these organs is intelligible through sexual selection, if the more odoriferous males are the most successful in winning the females, and in leaving offspring to inherit their gradually-perfected glands and odours."*
*Charles Darwin (1871)*

**Abstract:**

Chemical communication is central to reproductive and social signalling, territoriality and alarm signalling amongst mammals, with olfactory cues carrying chemical signals for age, sex, dominance and group or individual identity. African elephants use a multitude of odours during social and familial interactions, suggesting olfactory cues may mediate individual and herd recognition. Individual and kin discrimination through odour can serve important functions in group-living, social mammals as it aids in inbreeding avoidance and in facilitating reciprocal relationships between individuals and among relatives. Elephants, a natally philopatric, but polygynous species, live in multi-tiered, fission-fusion matriarchal societies. Previous research on kin recognition in elephants suggests that Asian elephant calves have a chemosensory signal for kinship in urine, while another study proposes that African elephant females can recognize up to thirty family members from their urine. Using preference tests, we show that African elephants discriminate between familiar and unfamiliar non-kin urine and dung odours. Elephants inspected the unfamiliar scent first, sniffed the unfamiliar scent significantly longer than familiar odours, and spent significantly more time investigating urine than dung. To explore potential differences in expressed metabolic features between adult male and female African elephant urine, volatile organic compounds (VOCs) were extracted from elephant urine using solid-phase microextraction (SPME) gas chromatography mass spectrometry (GC_MS), and the XCMS analysis platform applied. Results suggest that African elephants have a chemosensory signature in urine for sex, age and individual identity. Previously published phenols and pheromones (m-and p-cresol) were identified, in addition to alkan-2-ones and alkan-2-ols in male urine. Contrary to previous research, we putatively identified compounds associated with male elephants (2-Heptanone, 4-Heptanone, cyclohexanone) in adult females. These results provide evidence that elephants extract fixed and variable conspecific information from urine and dung, with compounds identified in elephant urine suggesting to play a role in eliciting behaviours in a number of mammals, including sexual attraction, oestrus, puberty, alarm recruitment, aggression and locomotory behaviour. These results contribute towards future research into the role of olfaction in elephant social organisation.

**Key words**: African elephant, GC_MS, kin recognition, olfaction, scent discrimination, dung, urine, XCMS.

## 4.1 Introduction

Olfaction is a primary mode of communication in most mammals. Mammals emit and receive chemical signals from conspecifics in a range of contexts, from foraging, kin discrimination, mate choice and reproduction, social relationships and parent-offspring recognition (Wyatt 2003). Olfactory signals in urine, faeces, glandular secretions and scent marks contain information related to species, age, sex, reproductive status, rank, health or identity (Setchell et al. 2010, Kean et al. 2017, Stoffel et al. 2015, Kent & Tang-Martinez 2014).

The ability to discriminate between familiar and unfamiliar conspecifics from odour may confer significant fitness advantages as familiarity may mediate conspecific interaction and behaviour that is critical to reproductive success. Kin discrimination, kin selection, social behaviour and intrasexual competition are mediated by olfactory cues in a number of species (Wyatt 2003) with olfactory discrimination mediating inbreeding avoidance, conflict, territorial maintenance, mate choice, dominance rank relationships, social cohesion, establishment of mutualistic relationships, intrasexual competition and parental care (Archie & Theis 2011, Clutton-Brock & Huchard 2013).

The ability to discriminate individual odours has been recorded in a diversity of species, including ring-tailed lemurs (*Lemur catta*), golden hamsters (*Mesocricetus auratus*), mole rats (*Cryptomys anselli*), ferrets ( *Mustelo fero*), raccoons (*Procyon lotor*), meerkats (*Suricata suricatta*), white rhino (*Ceratotherium simum*), penguins *(Spheniscus humboldti*) (Coffin et al. 2011), and kittiwakes (*Rissa tridactyla*) (Palagai & Dapporto 2006, Todrank & Heth 1996, Johnston et al. 1993, Kent & Tang-Martinez 2014, Marneweck et al. 2017, LeClaire et al. 2011). Studies have further shown that odour cues may underlie genetic relatedness (Odour-Gene Covariance or OGC), which animals are able to detect, even in unfamiliar kin (Boulet et al 2009, Heth et al. 2004, Heth et al. 1998, Heth et al. 2003). Sources of body odour include urine, faeces and specialized scent glands. In social mammals in particular, individual odours can allow conspecifics to discriminate between members of their own social group, and foreign conspecifics (Burgener et al. 2009). This can serve important social and sexual functions in group-living mammals as it aids in resource defense, offspring care and social cohesion, as well

as in inbreeding avoidance and in facilitating cooperation among relatives. Group-living social mammals that discriminate between in and out group members include another fission-fusion mammal that lives in female clans - the spotted hyena (*Crocuta crocuta)* (Burgener 2009), while colony differences have been mediated by scent in naked molerats (*Heterocephalus glaber*) (O'Rian & Jarvis 1997), fur seals (*Arctocephalus gazella*) (Stoffel et al. 2015), and bats (*Eptesicus fuscus*) (Bloss et al. 2002 ).

African elephants are an ideal model species to investigate olfaction in a variety of behavioural contexts. African elephants possess a large brain, enlarged neocortex, a highly developed olfactory system, 2000 olfactory receptor genes (Niimura et al. 2014) and unique and specialized scent glands (temporal gland). Conspecifics are greeted and investigated by sniffing and touching, blowing onto urine spots and reinhaling VOCs (Schulte et al. 2005), sniffing dung, inspecting genitalia, mouth and the temporal gland with distinct trunk-tip behaviours. Given signaller and receiver condition, olfactory molecules may be transferred from the trunk tip to the vomeronasal organ (VNO) or the palatal pits in the roof of the mouth for further chemosensory analysis.

African elephants are sexually dimorphic and experience fluid and dynamic systems of spatial use and grouping. Neither sex is territorial, and males and females live in separate but overlapping spheres and social systems. Female African elephants are philopatric and are closely bonded to their relatives: they live in tightly-knit matriarchal groups comprised of females and their offspring. Females invest highly in their offspring with extended maternal care. Females can breed throughout the year, and come into oestrus for a very brief 3-6 days (usually after the wet season when resources are abundant), only conceiving every 3-9 years (Moss 1988). Older larger females dominate smaller younger females, creating a linear hierarchy within a social group (Archie et al. 2006). Dominance hierarchies occur in the dry seasons, when resources are limited (Wittemyer et al. 2007). Their highly sophisticated fission-fusion systems are flexible and occur at multiple tiers of social organization (Wittemyer et al. 2005), fissioning into small herds or fusing into aggregations containing hundreds of animals in days, weeks or months. Greeting ceremonies among herds of African elephants are marked by intense urination, defecation, and temporal gland secretions (TGS). Although no empirical

studies have explained the function of these chemical emissions, it is likely that these individual odour signatures underlie kin recognition in elephants (Wyatt 2003) and could possibly promote bond group cohesion.

Based on studies in Asian elephants, the urinary pheromone Z7-12:Ac signals impending oestrus in elephant females during the preovulatory phase, providing an olfactory mechanism to attract musth bulls. Behavioural studies suggest that female African elephants send a similar olfactory signal to male elephants in urine, dung and genitalia (Poole & Moss 1989), yet the identity of the pheromone is yet to be confirmed (Bagley et al. 2006). Female elephants can detect when a conspecific is in oestrus from urine (Meyer et al. 2008), while males use the flehmen response in reaction to the oestrus pheromone (urine or genitalia) and assess musth/non-musth status from urine in other males. Rasmussen and Krishnamurphy's (2000) observations of captive Asian elephant calves and offspring seem to suggest that elephants have a chemosensory signal for kinship in urine. Calves live in intimate association with their mothers and closely related females (von Dürckheim *in review*). Elephant calves flehmen to urine by 3 months of age, and flehmen to the urine from their mothers twice as frequently as to urine from other females, both related and non-related. In studies of mother–offspring pairs that lived together and were later physically separated, offspring responded significantly more to maternal urine than to all other controls (Rasmussen 1995). Genetic signals in elephant semiochemicals are yet to be discovered, but elephants are socially complex, which with their long periods of social and olfactory learning, extraordinary memory, complex cranial morphology and brain size, are indicative of higher cognitive ability.

Females can track musth bulls by their urine trail, which encodes musth, and consequently, dominance, and urine dribbling during musth amounts to an astonishing 400 litres every 24 hours (Poole & Moss 1989). Males live solitary lives or in bachelor herds, dispersing from their natal herd at approximately 15 years of age. Males become sexually competitive after the age of 25, later than females who are sexually mature at approximately 15. Research on the male rutting period, known as musth, indicates elevated testosterone levels which are expressed in breath, urine and TGS in Asian and African bulls. The expression of musth, as a signal of dominance, contributes towards the 'smooth functioning of male elephant society" (Rasmussen,

et al. 2002). Musth in bigger, older bulls suppresses aggression and musth (reproductive output) in younger males (Slotow et al. 2000), with chemosignals in musth varying with age dominance and reproductive status (Rasmussen & Greenwood 2003). Musth in large bulls in Amboseli in Kenya is asynchronous, but overlapping, and can range from a few days to several months (Poole & Moss 1989). Musth bulls and oestrus cows are a rare occurrence in a widely spatially dispersed species, and musth bulls range widely in pursuit of a receptive female, losing body condition in the process. Males have been shown to be able to distinguish conspecific female urine collected at the time of ovulation from urine obtained at the mid-luteal time of the estrous cycle (Bagley et al. 2006). Musth bulls will also guard receptive cows, with receptive females preferring to mate with musth bulls, and studies have shown that group size in females is significantly larger in the presence of musth bulls irrespective if females are receptive (Poole & Moss 1989). The most extensive study on African elephants demonstrates inbreeding avoidance within the species, where bulls not only avoiding breeding with natal kin but also with paternal kin (Archie et al. 2007), suggesting that males may be using phenotype matching to identify kin. Phenotype matching has been demonstrated in other vertebrates, and the use of olfactory cues explored (Brown & Eklund 1994, Zelano & Edwards 2002). In elephants, research suggests that the signal may be olfactory, however it is unknown whether olfactory signals are closely correlated enough with relatedness to be sensitive cues for phenotype matching.

Urine is considered an important chemosignalling source in elephants. Studies have shown that Asian elephants can detect pheromones in urine, breath and temporal glands (Rasmussen & Krishnamurthy 2000, Rasmussen 1988, 1995, 1998, Rasmussen & Schulte 1998). In mature elephant males, frontalin is released in urine and TGS, evoking caution in female conspecifics and avoidance in younger males (Rasmussen & Greenwood 2003). Dung, urine and TGS of African elephants are deposited on elephant pathways and at waterholes within home ranges (Parker & Graham 2019, von Dürckheim 2011), and are used to assess reproductive and fitness status of conspecifics (Sukumar 2003, Parker & Graham 2019). Insect pheromones have been identified in the urine of female African elephants (Goodwin et al. 2006). Other compounds verified in African elephant female urine include pentanone, heptanone, octanol, 4-methylphenol

acetophenone, 4 ethylphenol (Goodwin et al. 2012), and the following alkan-2-ones and alkan-2-ols: pentan-2-one, heptan-2-one and octan-2-ol. Confirmed pheromones in African elephant females include, trypanthrine trans-nerolidol, p-cresol, and m-cresol (urine).

The social organization of this species, coupled with extensive behavioural observation, suggest that olfactory communication plays an important role in the behaviour of African elephants. The current study has shown that African elephant discriminate between individual humans from their body odour (von Dürckheim et al. 2018, Chapter 2), and that African elephants can track and discriminate between two human odour trails (von Dürckheim et al. *under review*, Chapter 3). The interspecific olfactory acuity demonstrated by the elephants raised the question whether African elephants can discriminate between the odour of individual conspecifics. The role of intra-individual recognition in elephants is poorly researched and Asian elephant experts have called for increased research in this field (Sukumar 2003). Unlocking chemicals underlying elephant behaviour can be applied to further zoological enrichment programs by the application of this knowledge to captive breeding efforts (Schulte et al. 2007), reduction in anxiety in captive and wild populations, mitigating human-elephant conflict, and to establish pregnancy in sanctuary or zoo environments. In wild populations, translocations and resettlement of elephants can be further improved by applying knowledge of how elephants use scent for territory settlement, establishing social relationships and mating.

The objective of this study was to determine whether African elephants could discriminate individual differences in urinary and fecal odours of conspecifics, and to establish whether individual urinary profiles are chemically distinct. The chemical composition of urine is described, and key compounds compared with published libraries such as NIST. This study evaluates whether a) elephants spend more time investigating unfamiliar conspecific odours, b) odour profiles would differ between age and sex, and c) urinary chemical profiles would be more similar among highly related adults.

## 4.2 Methods:

**4.2.1 Subjects:** This research used six elephants *(L. africana*) from Adventures with Elephants (AWE) in Bela-Bela, South Africa, for behavioural biosassays. Of the six elephants, only the calf

was born in captivity. Elephants are well habituated to people, and have been successfully utilised for scientific research on elephant morphology, scent detection, hormones and biochemistry, laterality, olfaction, Social Network Analysis, and infrasound communication (von Dürckheim et al. 2018, Panagiotopolou et al. 2016, Miller et al. 2015, Zeppelzauer et al. 2014, Stoeger et al. 2012).

**Table 4.1.** Summary of the main characteristics of the six elephants analysed (a and b indicate mother calf units). * Pregnant ** GnRH

| Name | Abbreviation | Sex | Year of birth | Year of capture | Rank | Age |
|---|---|---|---|---|---|---|
| Chova | CHO | Male | 1995 | 2007 | 4 | ~24 |
| Chishuru ** | CHI | Male | 1997 | 2008 | 5 | ~22 |
| Shan (a) | SH | Female | 2000 | 2008 | 2 | ~18 |
| Nuanedi | NU | Female | 2001 | 2008 | 3 | ~17 |
| Mussina* | MU | Female | 2001 | 2008 | 1 | ~17 |
| Zambezi (b) | ZA | Male | 2015 | born at reserve | 6 | 3 |

**4.2.2. Ethics statement:** Research protocols were cleared by USAMRMC (US Army Medical Research and Materiel Command) Animal Care and Use Review Office (ACURO) and by Stellenbosch University IACUC (The Institutional Animal Care and Use Committee). Stellenbosch University Ethics Approval Protocol Number: SU-ACUM15-00002.

**4.2.3 Behavioural bioassay:** African elephants engage in prolific urination and defection on a daily basis. Previous work has shown that African elephants monitor conspecifics from urine (Bates et al. 2008). Twelve elephants from separate locations (AWE elephants as the “in-group”, and six elephants from a separate location as the “out-group” – please refer to elephant outgroup details in Appendix, Table S 4.2.3) were sampled and 100ml urine and two dung boli collected from each individual. Handlers wore latex gloves at all times, and samples were taken at the same time each morning between 8-9am. Faeces were collected and placed in small plastic containers and frozen at -20°C. Urine was collected directly mid-stream where possible, in a glass jar, then sealed and frozen at -20°C. All odours used had the same exposure period between deposition in the glass jar and introduction to the subject elephant.

**4.2.4 Experimental procedure:** To test whether African elephants discriminate conspecifics via olfactory cues using prior association, urine and dung from familiar (in-group) and unfamiliar (outgroup, from a different location) conspecifics were presented to five adult elephants (Table

4.1). Prior familiarity between elephants of the “in-group” and elephants in the “out-group” was established from historical data on each elephant’s capture site and translocation history, while relatedness was established by DNA analyses. Preference tests for urine samples were conducted at a controlled site at AWE. Twenty ml of familiar (in-group) and unfamiliar (out-group) urine was placed into labelled buckets for ease of sample identification. Buckets for each test were only used once. Preference tests for dung were conducted in an open field as samples were readily identifiable from a distance.

For each test, two odour samples were thawed an hour prior to testing. Donors of each pair of odours were the same sex. Elephants were individually called by a handler, and the elephant was free to explore the scent sample for 60secs. For each test, elephant behavioural response was filmed, sniffing duration timed with a stopwatch and any possible infrasound recorded by third parties blind to the type of sample. Signaller/receiver odour pairs were unique and only used once on the subject elephant.

**4.2.5 Sample collection:** Samples were collected on the same day at 10am. Midstream urine was collected directly into a 100ml glass jar, and subsequently frozen at -20°C. Dung boli were halved and stored in a frozen plastic container at -20°C.

**4.2.6 GC_MS analyses:** GC-MS analyses were carried out on an Agilent 6890N Gas Chromatograph coupled to a 5975B Mass spectrometer with a splitless injection and a Zebron capillary ZB-WAX column (30m, 250µm, 0.25 µm) using a Supelco 65µm PDMS/DVB, Stableflex, 23Ga SPME (solid phase micro extraction) fiber for headspace analysis. The pre-incubation time of the fiber was 300s at 50°C with an agitator speed of 250rpm. Extraction time was 1800s, with a desorption time of 600s. The scan range of the mass spectrometer was 35 - 550m/z, with the analysis program starting the oven at 40°C which was held for five minutes, the temperature was then ramped at 5°C/minutes to a final temperature of 240°C which was held for two minutes. Semiochemicals that had consistent retention times and accounted for > 0.05% of the area of the total chromatogram were retained. To align the peaks between

chromatograms, semiochemical retention times (rt) were standardized against retention times of two standards: Anisole D8 (rt= 14.83mins) and 3-Octanol (rt = 16.29mins).

The samples were transferred into a 20mL SPME headspace vial and sealed with a polytetrafluoroethylene (PTFE, Teflon®)/silicone septa and steel cap with 50 µL of Anisole d8 added as internal standard. Vials were equilibrated at 30°C for 5mins using a CombiPAL solid-phase microextraction (SPME) autosampler (CTC, Switzerland). A conditioned (conditioned by heating in a gas chromatograph injection port at 20°C for 60mins) fibre coated with a 50/30µmm thickness of divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS), was inserted into the headspace above the sample and held for 30mins (with agitation). The fibre was consequently withdrawn into the needle by the autosampler and inserted into the injection port of a gas chromatograph (GC) 6890N (Agilent Technologies, Palo Alto, CA, USA) coupled with a mass spectrometer detector 5975B (Agilent Technologies). The SPME fibre was desorbed and held in the injection port (250°C) for ten minutes. The fiber was inserted in a fibre conditioning station for 10mins between samples for cleaning to prevent cross-contamination. The injection port was operated in pulsed splitless mode. Volatile compounds were separated using a polar ZB-Wax capillary column (30m, 0.25mm i.d. 0.25µm film thickness). The oven temperature was initially held at 40°C for five minutes and increased to 240°C at 5°C/mins (held for 3mins). The total run time was 48mins. Helium was used as the carrier gas with a constant flow rate of 1.9mL/min. The transfer line was maintained at 280°C. The mass spectra were obtained using a mass selective detector working in electronic impact at 70 eV, operated in full scan mode (35-450m/z) with both the ion source and quadrupole temperatures were maintained at 240°C and 150°C, respectively.

**4.2.7 Chemometric data analysis:** After GCMS analysis, raw data were converted to netCDF files using MASSTransit software (by Palisade-Scientific Instrument Services, Ringoes, NJ, USA). GCMS spectra processing were carried out using freely available XCMS software (online version 1.21.01, The Scripps Center for Metabolomics, La Jolla, CA, USA) (Smith et al. 2006). XCMS Online is a user-friendly cloudbased informatics platform designed to process and visualize mass-spectrometry based, untargeted metabolomics data, providing comprehensive

statistical output and interactive visualisations. It provides a comprehensive solution from raw data processing to metabolite assignment through integration with the METLIN database (Gowda et al. 2014, Tautenhahn et al. 2012, Huan et al. 2017). A recent study on epilepsy biomarkers in mice urine (Fujita et al. 2019) and on skin biomarkers for cancer (Abaffy et al. 2013) successfully utilized SPME GC_MS in combination with XCMS in identifying urinary volatile organic compounds. The default parameters for GC Single Quadruple were applied [general: GC/single quad MS (matched Filter), GC-EI, single quadruple MS, retention time (RT) format: minutes, polarity: positive, feature detection: matched Filter, step: 0.25, FWHM: 3, RT correction: method: peak groups, nonlinear/ linear alignment: loess, extra: 1, missing: 1 Alignment: mzwid: 0.25, minfrac: 0.5, bw: 10). GCMS spectra processing included: filtering and identifying peaks, matching peaks across samples, peak RT alignment and arranging peaks into peak intensity table for further statistical analysis. Thus, all chromatograms were simultaneously analyzed with identical settings. Two-group tests were selected to determine the metabolite features whose levels were significantly different between male and female elephants.

### 4.2.8 Statistical framework:

**4.2.8.1 Behavioural bioassays:** We used a two-way mixed model ANOVA as some subject elephants were tested more than once on urine from different elephants. Two tailed binomial tests determined whether there were differences in which sample the elephant investigated first. Despite the limitations in the number of elephants that could be tested, the statistics are robust enough to detect discrimination between odour types (R package ‘lmerTest’ version 3.1-3).

**4.2.8.2 Statistical Analysis Framework:** The computer code for R (GCAlign) was adopted from Ottensman et al. (2018) for alignment and normalisation of peaks, a package specifically developed for ecological and evolutionary research evaluating similarity patterns across multiple and variable biological samples. Peaks that were present in only one sample were excluded, which left a total of 526 retained substances.

**4.2.8.3 Overall patterns of chemical similarity:** Chemical profiles were visualized using multidimensional (MDS) scaling ordination and nonmetric multidimensional scaling (nMDS) based on Bray-Curtis Similarity Values within the XCMS online platform (online version 1.21.01,

The Scripps Center for Metabolomics, La Jolla, CA, USA). Each point in the 2-dimensional MDS plot represents an individual elephant swab, with clumped points representing individuals with greater chemical similarities. MDS has been successfully used in other studies to visualize chemographic data in mammals (Morelli et al. 2013).

**4.2.9 Identification of compound type:** The type of compound represented by each peak was identified by filtering compounds by quality. Compounds were retained that had a probability match of >70%. Identification of putative substances was based on their retention times, and by comparing their mass spectra with the best match of the library of the National Institute of Standards and Technology (Gaithersburg, MD, USA). Compounds were categorized into ketones, phenols, esters, aldehydes, hydrocarbons and in known substances. Exact identification of each compound, through injection of commercial standards, was considered unimportant for the present study (as in Scordato et al. 2007).

**4.2.10 Genetics:** Blood was taken from the auricular vein of the elephants by a wildlife veterinarian using five x 4 ml sterile EDTA tubes with vacutainers and frozen at -20oC. Genomic DNA was extracted from whole blood for 12 individuals using the Prepfiler Automated Forensic DNA extraction kit (Thermo Scientific) and purified on the Kingfisher Flex Purification System (Thermo Scientific). Individuals were genotyped at 18 microsatellite loci using two multiplex panels that comprise previously reported loci from Archie et al. (2003), Comstock et al. (2000) and Eggert et al. (2003). For each multiplex panel PCRs and electrophoresis were performed with 10µl reactions using the KAPA2G™ Fast Multiplex PCR Kit (Kapa Biosystems). Amplification PCRs were performed on a GeneAmp PCR System 9800 as follows: 95°C for 3min; 30 cycles of 95°C for 15sec, 60°C for 15sec and 72°C for 30sec; and a final amplification at 72°C for 10 min. Electrophoresis was performed on a 3500x Genetic Analyzer (Thermo Scientific) and the resulting data were analysed in STRand (Toonen & Hughes 2001) using the GeneScan™ 500 LIZ® size standard (Thermo Scientific). To estimate pairwise genetic relatedness between individuals, the software Identix (Belkhir et al. 2002)was used to calculate Queller and Goodnight's RQG (Queller & Goodnight 1989) for each dyad across the data set (please refer to the Supplementary Information S4.1).

## 4.3 Results:

**4.3.1 Subjects:** Five adult African elephants were included in this study, including two adult males, and three adult females. Shan was suckling her 3-year old calf Zambezi, Mussina was pregnant and Nuanedi was nulliparous. Chishuru is presently treated with Gonadotrophin releasing hormone (GnRH) to prevent inbreeding with the females, while Chova is untreated (Table 4.1).

**4.3.2 Behavioural bioassays**: During the preference test, the five elephants first investigated the odour from unfamiliar conspecifics (Figure 4.1). The average duration the subject elephant spent sniffing the urine and dung of an unfamiliar conspecific was significantly higher (Figure 4.2) than the time spent sniffing urine and dung of familiar conspecific ($p<0.01$). The difference in sniffing duration between familiar and unfamiliar urine tended to be more significant in males. Mean investigation time in male elephants for unfamiliar urine was twenty six seconds, and eleven seconds for female elephants ($p <0.03$). No sex effect could be found for the dung preference test, with subjects spending similar times investigating familiar and unfamiliar boli.

Both females and males spent less than eight seconds investigating urine and dung odours from familiar conspecifics. When assessing the urine of unfamiliar conspecifics, subjects of both sexes spent twice as long investigating the olfactory signal. For male elephants, mean investigation time for urine was 34 seconds, while for dung it was 11.5 seconds. For females, mean investigation time for urine was 24 seconds, and for dung 12 seconds. Although sniffing duration may appear brief in terms of seconds spent, this is not unusual as similar studies recorded short sniffing durations in olfactory discrimination trials in raccoons (Kent & Martinez 2014).

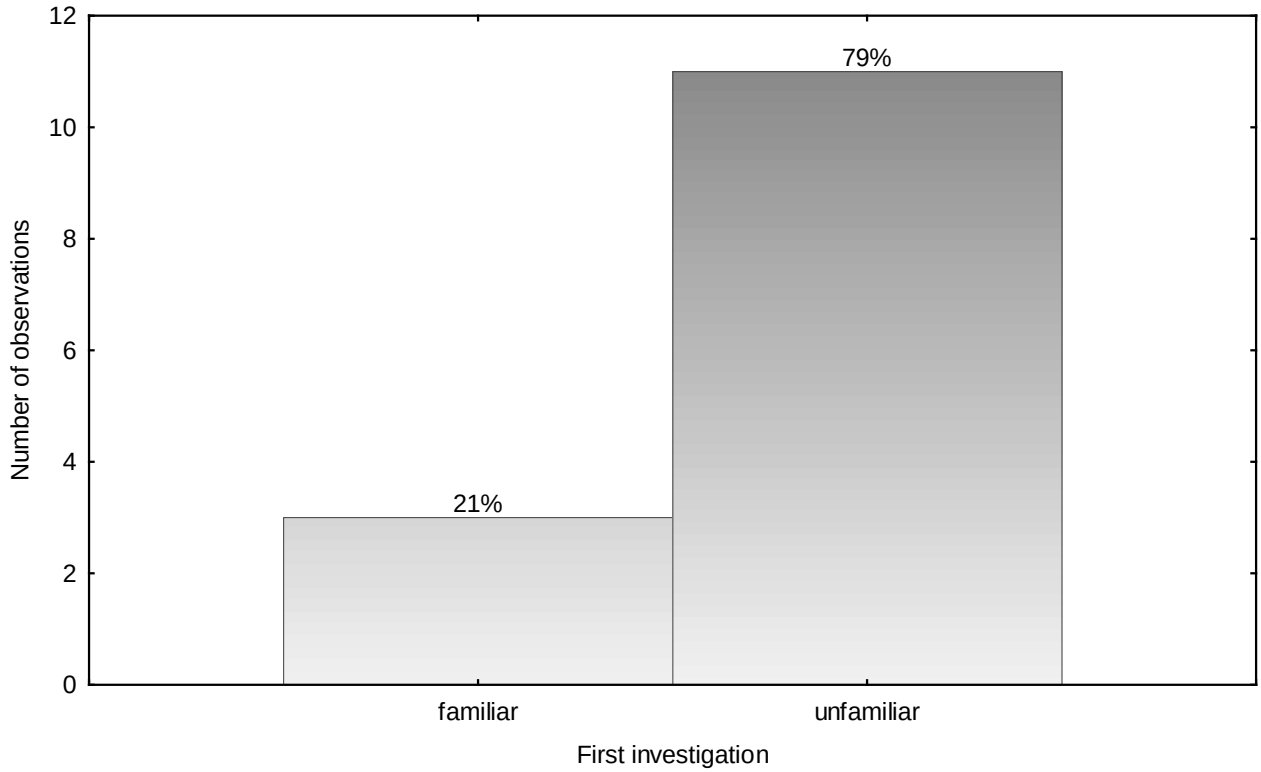
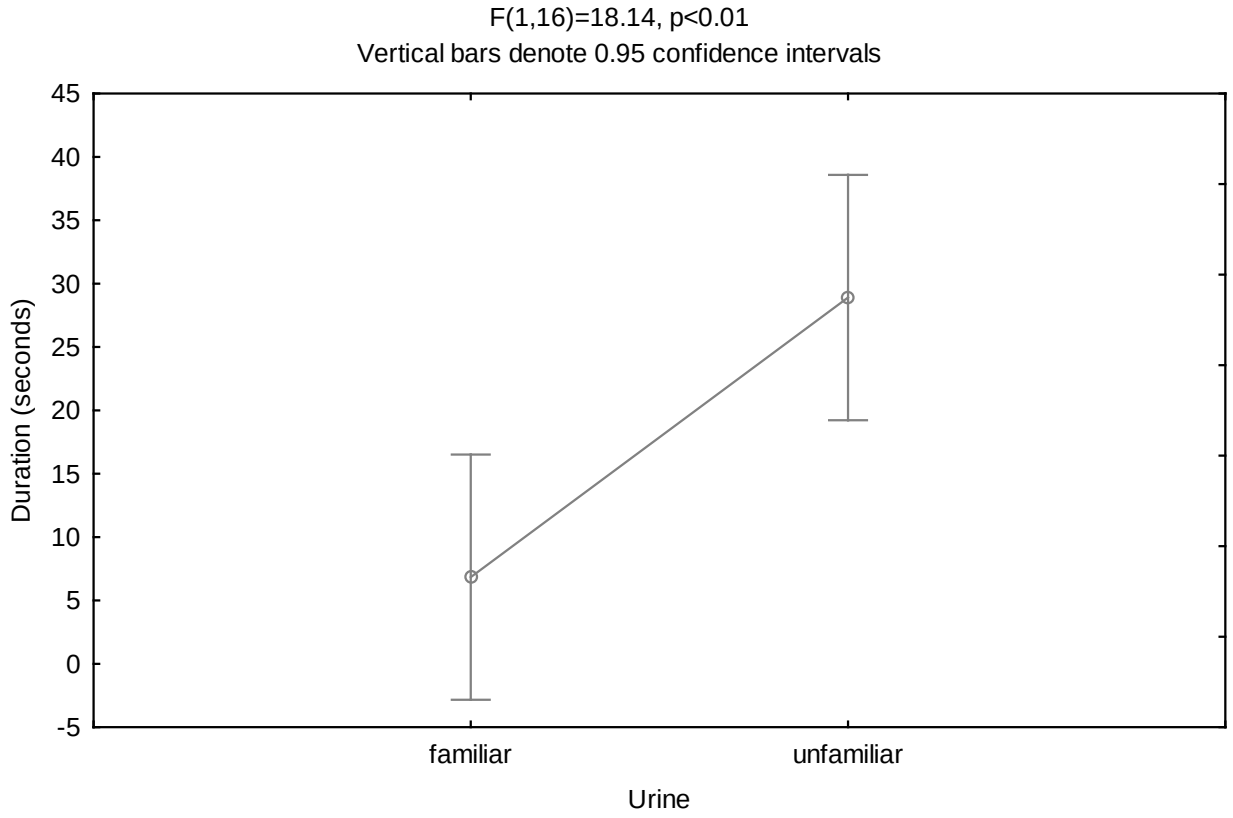



**Figure 4.1:** Elephants first investigated the unfamiliar odour.



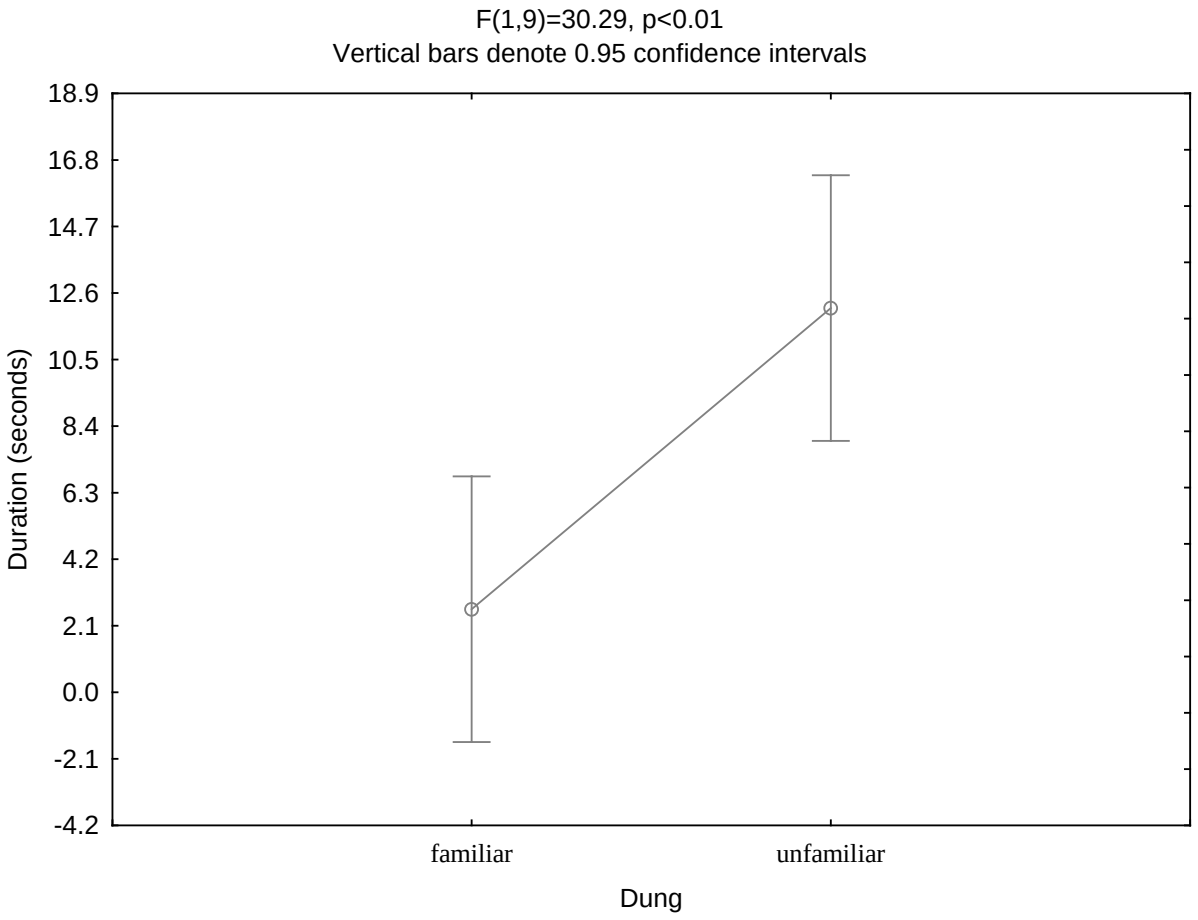




**Figure 4.2**: Elephants showed discrimination between familiar (in-group) and unfamiliar (out group) urinary and dung odours.

**4.3.3. Comparison of male and female African elephant urine samples:** We analysed three female against three male urine samples. Volatile collection was matched, as samples were collected on the same day and at the same time. Typical SPME GC_MS TIC chromatograms of urine samples of African elephant males and females are shown in Figures 4.4 and 4.5. Through visual inspection of the chromatograms, the chemical composition of the three males and three females appear individually distinctive. Difference among the males were quantitative, with peak strength varying between the two adult males, as well as qualitative (identity of chemicals) – the subset of chemicals present in the male calf's urine (Zambezi). The three adult females' urinary chromatograms appeared more similar, with peak strengths quantitatively different across chemical signatures.

As the focus of the study was the similarities/dissimilarities between male and female elephant urine, MDS and PCA metabolomics visualisation tools in the XCMS analytics platform were used to express differentially expressed metabolic features. It is clear that urinary profiles separated into two groups – male and female. The MDS was rerun excluding Zambezi's data as the male calf's olfactory profile appeared markedly different to that of the adults. Results remained stable with good separation between the sexes (Figure 4.6). Results from XCMS analysis, the mirror plot, summarises the most significant features-ions using threshold of p >0.01 and fold change >1.5 is presented in Figure 4.7. A total of 29 metabolic features were identified that were significantly different between adult male and female urine samples (Table S4.2 in Supplementary Information)

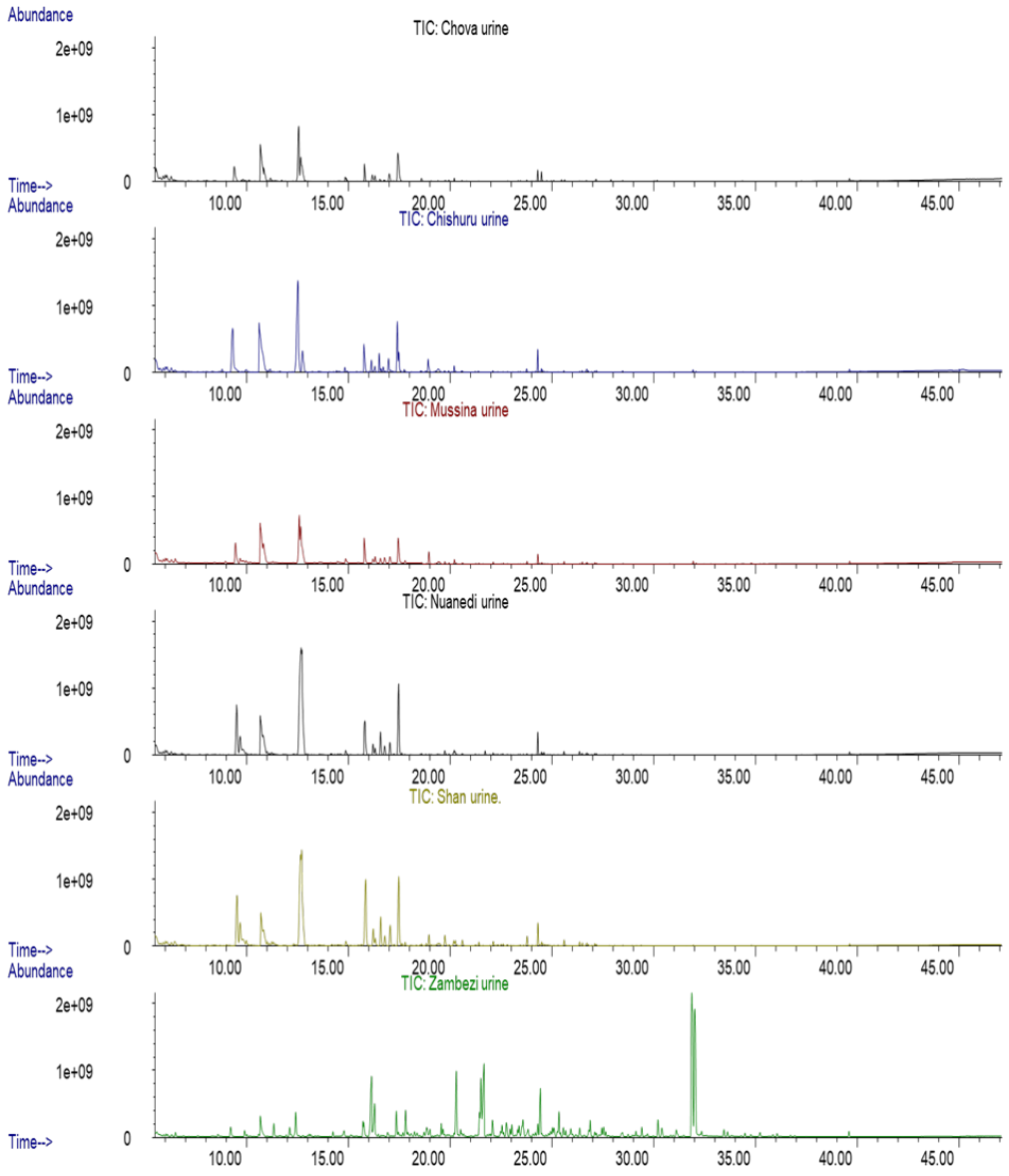



**Figure 4.3:** Representative gas chromatograms of urine of all African elephants

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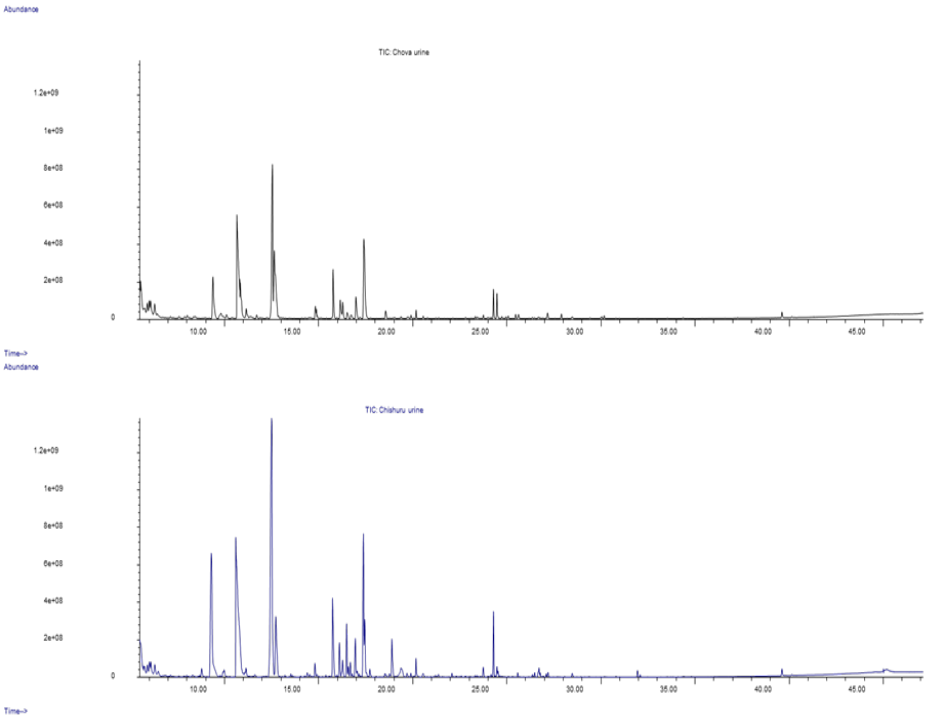



**Figure 4.4:** Representative gas chromatograms of urine of individual adult male African elephants (a) Chova and (b) Chishuru.

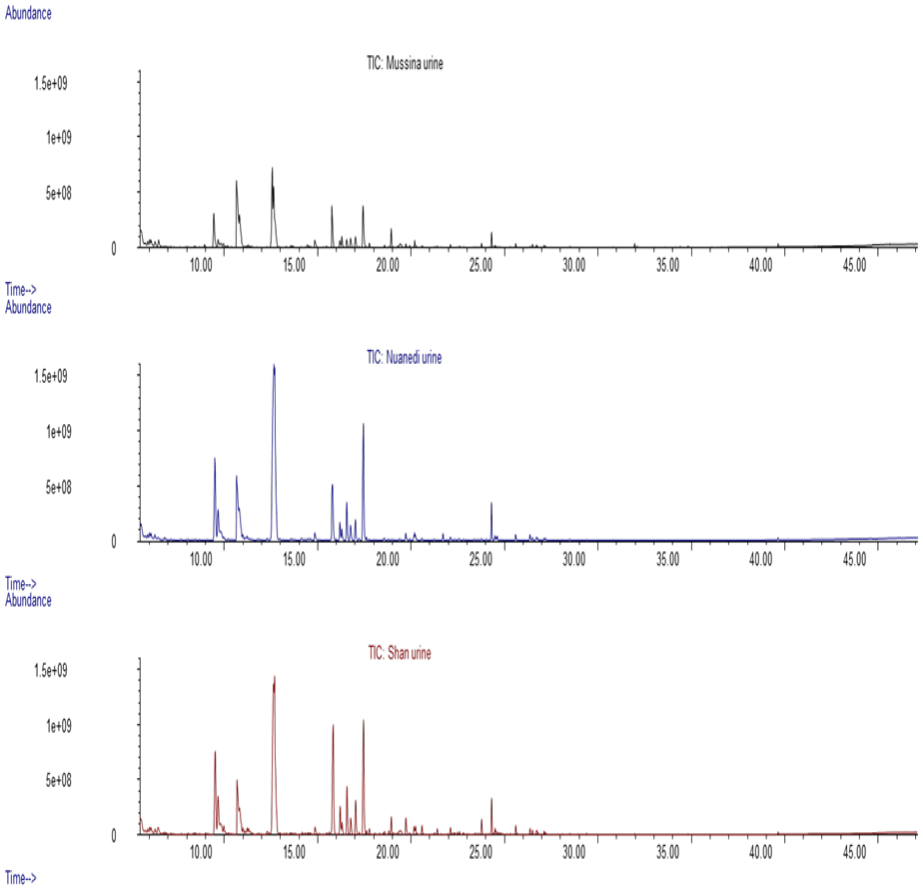
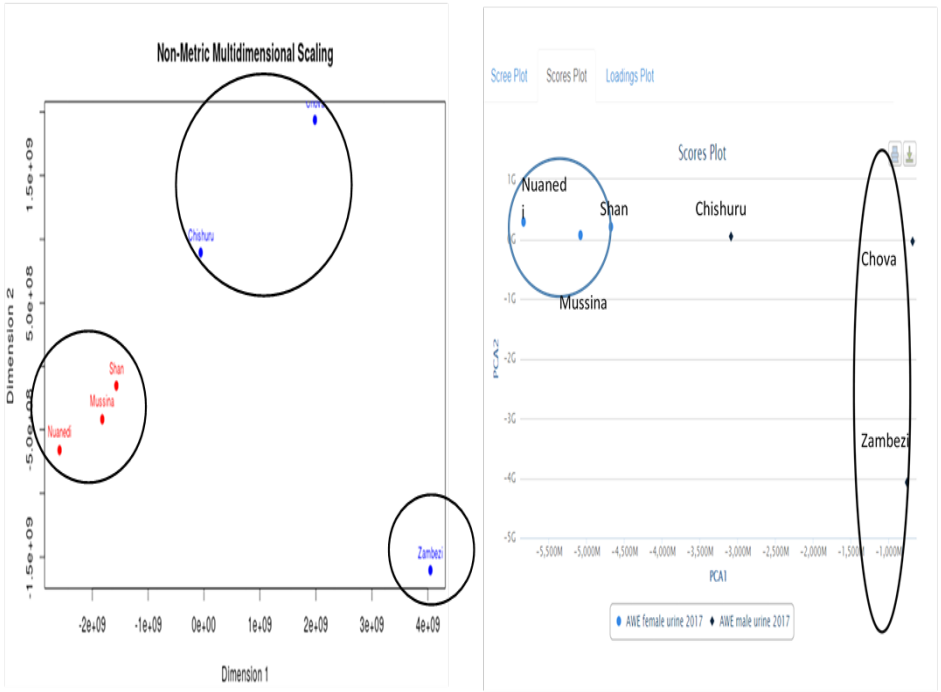



**Figure 4.5**: Representative gas chromatograms of urine of adult female African elephants (a) Mussina (b) Nuanedi and (c) Shan



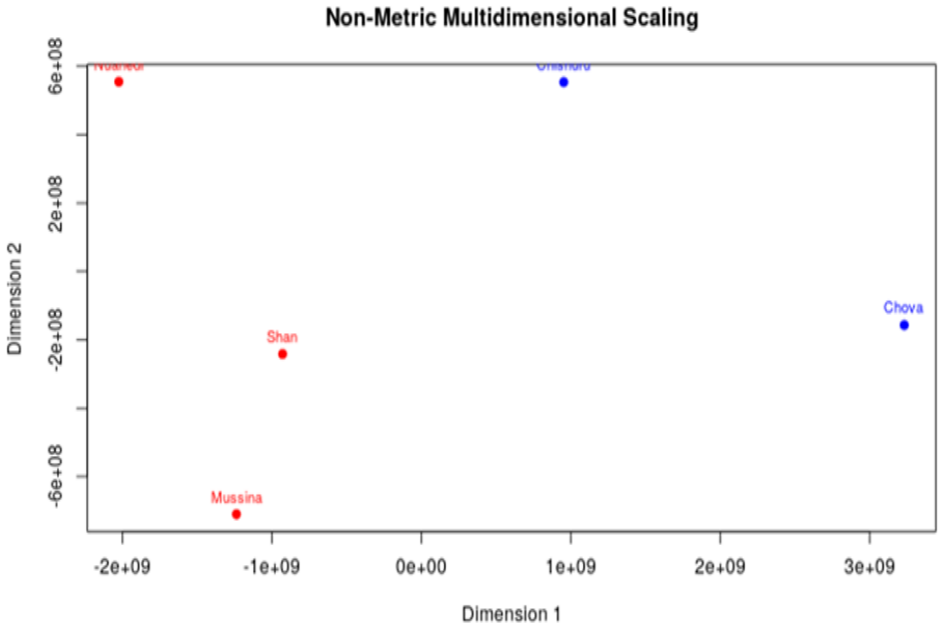




**Figure 4.6:** Non-metric multidimensional scaling (nMDS) analysis of urinary chemical compounds showing separation by age (top left) and by sex (bottom). A Scores plot (top right) shows the correlation between the samples.

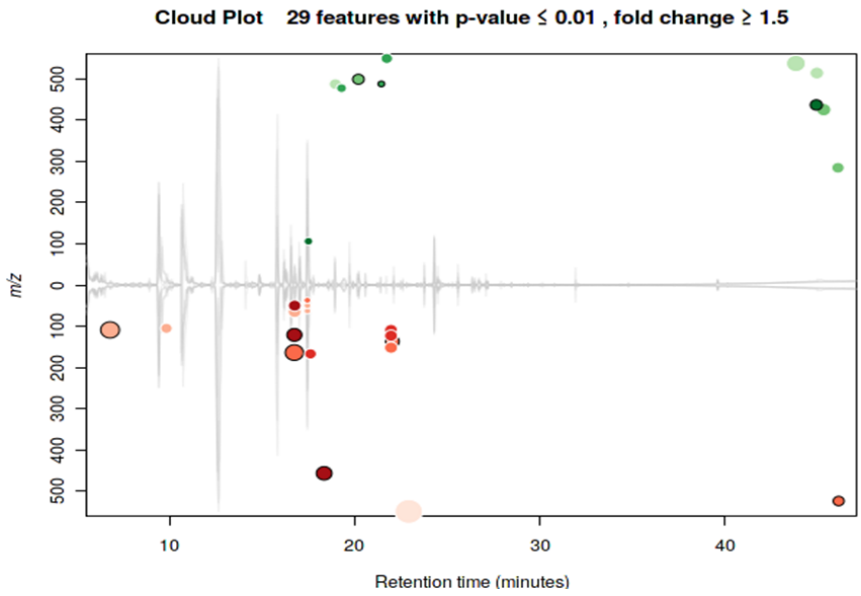



**Figure 4.7:** XCMS analysis. Mirror plot showing up (green circles) and down (red circles) regulated metabolic features ions between adult male and female African elephant urine samples. The size of each circle corresponds to the (log) fold change of the feature, while the intensity of the colour is used to represent the p value with brighter circles having lower p-values i.e. higher significant differences (Tautenhahn et al. 2012).

### 4.3.4 Determination of urinary volatiles of elephants using NIST:

Elephant urine contained more ether compounds than TGS, buccal secretions and genital secretions (refer to subsequent chapters), yet less aldehydes than TGS, buccal and genital secretions. Compounds included a variety of chemical structures including phenols, ketones, aromatic compounds and arenes, ethers, alcohol, alkenes and alkanes (S4.3). In the adult females, ethers and aromatic compounds predominated, followed by arenes.

**4.3.4.1 Males (Table 4.2)**: As was the case with all adult females, both adult males had 4-Heptanone in their urine. Benzoic acid, benzene, propanoic acid and 9-octadecanoic acid (Z) methyl was also identified. Chova, the breeding and dominant male, had a very high number of arenes and aromatic compounds, including Hexaoxaaicosane. A confirmed pheromone, exo-brevicomin (Goodwin et al. 2006), was identified in Chova's urine, but not in the other male. Chova's male calf, Zambezi, is the only elephant that has phenols as a major compound class in his urine. Chishuru, who is treated with GNRH, had a significantly high number of alcohols, including ergosterol, phenylethyl alcohol and 3-octanol, and fewer ether compounds than the other elephants. Benzaldehyde was found in both males, while 2-propanal was only found in Chova, the breeding male. Chishuru, the GNRH treated male, had many ketones in his urine like the females, but differing in ketone identity (4-Heptanone, 2-methyl, cyclohexanone, 3-Buten-2-one, 2-Buten1-one, and 2-Cycleohexen-1-one,2-methyl-5-. The phenols, m-cresol and p-cresol (confirmed pheromones in elephants) were also identified in male urine. A monoterpene, namely Alpha Phellandrene, a confirmed pheromone in blackberry aphids and European badgers, was unique to Chishuru's urine. A terpenoid – y-terpinene (or gamma terpinene) was present in all elephants except Mussina, the pregnant female. This compound is a confirmed pheromone in bark beetles and European rabbits and a discussion point.

**Table 4.2:** Volatile organic compounds (>70% NIST match), and the class of chemicals they belong to, detected in the urine of male African elephants.

| | Compound | Compound category |
|---|---|---|
| 1 | 1,2-Ethanediol, 1,2-diphenyl- | Alcohol |
| 2 | 1,6-Octadien-3-ol, 3,7-dimethyl- | Alcohol |
| 3 | 1,7,7-Trim+D2:E56ethylbicyclo[2.2.1]hept-5-en-2-ol | Alcohol |
| 4 | 1-Nonanol ** | Alcohol |
| 5 | **1-Octen-3-ol * **** | Alcohol |
| 6 | 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)- | Alcohol |

| | Compound | Compound category |
|---|---|---|
| 7 | **2-Decanol** | Alcohol |
| 8 | 2-Dodecanol | Alcohol |
| 9 | 2-Methyl-2-butyl-1,3-benzodioxole | Alcohol |
| 10 | **2-Nonanol **** | Alcohol |
| 11 | 2-Tetradecanol | Alcohol |
| 12 | **2-Undecanol** | Alcohol |
| 13 | 3-Cyclohexene-1-ethanol, .beta.4-dimethyl- | Alcohol |
| 14 | **3-Methyl-2-(3-methylpentyl)-3-buten-1-ol** | Alcohol |
| 15 | 3-Methylbenzyl alcohol | Alcohol |
| 16 | 3-Octanol | Alcohol |
| 17 | 5-Ethyl-3-methylhept-1-en-4-ol | Alcohol |
| 18 | 5-Octen-2-yn-4-ol | Alcohol |
| 19 | 6-Octen-1-ol, 3,7-dimethyl-, (R)- | Alcohol |
| 20 | Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-dimethyl- | Alcohol |
| 21 | Cyclohexanol, 2-[2-pyridyl]- | Alcohol |
| 22 | E-11,13-Tetradecadien-1-ol | Alcohol |
| 23 | Tricyclo[4.4.0.0(2,7)]dec-3-ene-3-methanol, 1-methyl-8-(1-methylethyl)- | Alcohol |
| 24 | 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis- | Alcohol |
| 25 | **Benzaldehyde *** | Aldehyde |
| 26 | **Decanal *** | Aldehyde |
| 27 | 1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl- | Aldehyde |
| 28 | 1-Methyltricyclo[2.2.1.0(2,6)]heptane | Alkane |
| 29 | Bicyclo[3.1.1]heptane, 6,6-dimethyl-3-methylene- | Alkane |
| 30 | Cyclododecane | Alkane |
| 31 | Cyclooctane | Alkane |
| 32 | Cyclopentadecane | Alkane |
| 33 | Cyclopentane, 2-methyl-1-methylene-3-(1-methylethenyl)- | Alkane |
| 34 | Cyclopropane, 1,1-dimethyl-2-(3-methyl-1,3-butadienyl)- | Alkane |
| 35 | Cyclopropane, nonyl- | Alkane |
| 36 | Decane, 2-methyl- * | Alkane |
| 37 | Decane, 3-methyl- | Alkane |
| 38 | Decane, 4-methyl- | Alkane |
| 39 | Decane, 5,6-bis(2,2-dimethylpropylidene)-, (Z,Z)- | Alkane |
| 40 | Decane, 5-methyl- | Alkane |
| 41 | Nonadecane | Alkane |
| 42 | Pentacosane | Alkane |
| 43 | Undecane * | Alkane |
| 44 | Undecane, 4,6-dimethyl- | Alkane |
| 45 | (-)-Neoclovene-(I), dihydro- | Alkane |
| 46 | 1,3,5,7-Cyclooctatetraene | Alkene |
| 47 | 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)- | Alkene |
| 48 | 1,5,9-Decatriene, 2,3,5,8-tetramethyl- | Alkene |
| 49 | 1,6-Dimethylhepta-1,3,5-triene | Alkene |
| 50 | 1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, | Alkene |
| 51 | 2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)- | Alkene |
| 52 | 2,6-Dimethyl-1,3,5,7-octatetraene, E,E- | Alkene |
| 53 | 5-Dodecene, (Z)- | Alkene |
| 54 | Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)- | Alkene |

| | Compound | Compound category |
|---|---|---|
| 55 | Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl- | Alkene |
| 56 | Bicyclo[4.2.0]octa-1,3,5-triene | Alkene |
| 57 | Cyclohexene, 1-methyl-4-(1-methylethylidene)- | Alkene |
| 58 | Cyclohexene, 3-methyl-6-(1-methylethylidene)- | Alkene |
| 59 | Cyclohexene, 4-methylene-1-(1-methylethyl)- | Alkene |
| 60 | Cyclopentene, 3-isopropenyl-5,5-dimethyl- | Alkene |
| 61 | Cycloundecene, 1-methyl- | Alkene |
| 62 | 3-Octadecyne | Alkyne |
| 63 | 9-Octadecyne | Alkyne |
| 64 | 2-Phenylcyclopropionamide, N-(4-phenylazo)phenyl- | Amide |
| 65 | Aniline | Amine |
| 66 | p-Aminotoluene | Amine |
| 67 | (1-Methylpenta-2,4-dienyl)benzene | Arene |
| 68 | .beta.-Chlordene | Arene |
| 69 | .delta.-Selinene | Arene |
| 70 | 1H-Indene, 1-methylene- | Arene |
| 71 | Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.7.alpha.8a.beta.)]- | Arene |
| 72 | Azulene, 1,4-dimethyl-7-(1-methylethyl)- | Arene |
| 73 | o-Isopropenyltoluene | Arene |
| 74 | o-Xylene | Arene |
| 75 | p-Xylene | Arene |
| 76 | Styrene | Arene |
| 77 | Biphenyl | Aromatic compound |
| 78 | Pentadecanoic acid** | Carboxylic acid |
| 79 | **Furan, 2-methyl-5**-(1,1,5-trimethyl-5-hexenyl)- | Cyclic ether |
| 80 | 9-Octadecenoic acid (Z)-, methyl ester | Ester |
| 81 | **Benzoic acid *** | Ester |
| 82 | Butanoic acid, 2,3-dimethyl-, methyl ester | Ester |
| 83 | Cyclohexanecarboxylic acid, methyl ester | Ester |
| 84 | Cyclopentanetridecanoic acid, methyl ester | Ester |
| 85 | Decanoic acid, methyl ester** | Ester |
| 86 | Diethyl Phthalate | Ester |
| 87 | Dimethyl phthalate | Ester |
| 88 | Heptanoic acid, methyl ester * | Ester |
| 89 | Hexanethioic acid, S-methyl ester | Ester |
| 90 | **Hexanoic acid, methyl ester *** | Ester |
| 91 | **Octanoic acid,** methyl ester * ** | Ester |
| 92 | Pentanoic acid, methyl ester * | Ester |
| 93 | Tridecanoic acid, 3-methyl-, methyl ester | Ester |
| 94 | Undecanoic acid, 2,6,10-trimethyl-, methyl ester ** | Ester |
| 95 | 1,4,7,10,13,16-Hexaoxacyclooctadecane | Ether |
| 96 | Pentaethylene glycol monododecyl ether | Ether |
| 97 | Tetraethylene glycol monododecyl ether | Ether |
| 98 | **2-Buten-1-one**, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- | Ketone |
| 99 | **2-Decanone** | Ketone |
| 100 | **2-Dodecanone** | Ketone |
| 101 | **2-Heptanone** | Ketone |
| 102 | 2H-Inden-2-one, octahydro-3a-methyl-, cis- | Ketone |

| | Compound | Compound category |
|---|---|---|
| 103 | **2-Octanone **** | Ketone |
| 104 | **2-Tridecanone** * | Ketone |
| 105 | **2-Undecanone** ** | Ketone |
| 106 | 3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (E)- | Ketone |
| 107 | **3-Octanone** | Ketone |
| 108 | 3-Octen-2-one | Ketone |
| 109 | 3-Undecanone | Ketone |
| 110 | **4-Heptanone** | Ketone |
| 111 | 4-Heptanone, 2-methyl- | Ketone |
| 112 | 4-Octanone | Ketone |
| 113 | 4-Undecanone | Ketone |
| 114 | 5,9-Undecadien-2-one, 6,10-dimethyl-, (E)- | Ketone |
| 115 | **Acetophenone** * | Ketone |
| 116 | **Cyclohexanone**, 2-(1-methylethylidene)- | Ketone |
| 117 | .alpha.-Phellandrene | Monoterpene |
| 118 | 1,3,8-p-Menthatriene | Monoterpene |
| 119 | D-Limonene | Monoterpene |
| 120 | **Disulfide, dimethyl** | Organosulfur |
| 121 | 2-Methoxy-5-methylphenol | Phenol |
| 122 | 3-Methyl-4-isopropylphenol | Phenol |
| 123 | Phenol, 2,3,5,6-tetramethyl- | Phenol |
| 124 | Phenol, 2,6-dimethyl- | Phenol |
| 125 | Phenol, 2-bromo-4-methyl- | Phenol |
| 126 | Phenol, 2-butyl- | Phenol |
| 127 | Phenol, 2-methoxy- | Phenol |
| 128 | Phenol, 2-methoxy-4-methyl- | Phenol |
| 129 | Phenol, 2-methyl-5-(1-methylethyl)- | Phenol |
| 130 | Phenol, 2-propyl- | Phenol |
| 131 | Phenol, 3-(1,1-dimethylethyl)-4-methoxy- | Phenol |
| 132 | Phenol, 3,4-dimethyl- | Phenol |
| 133 | **Phenol, 3-methyl- *** | Phenol |
| 134 | Phenol, 4-ethyl-2-methoxy- | Phenol |
| 135 | **Phenol, 4-methyl- * **** | Phenol |
| 136 | Phenol, m-tert-butyl- | Phenol |
| 137 | Phenol, p-tert-butyl- | Phenol |
| 138 | 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- | Terpenoid |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012), * confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

**4.3.4.2 Females (Table 4.3):** Female urine tended to contain more compounds than male urine (Mann-Whitney U, $p<0.08$). Female urine contained a mean number of 382 compounds, and males 267. For compound category contribution to overall urinary chemical profile for each elephant, please refer to supplementary information.

*Pregnant female*: Ketones identified by GC_MS included 3 Buten-1-one, 2-Nonen-4-one and Acetophenone, isobutyric acid, 3-Octanol, benzoic acid, crown ethers, furans, 3-Buten-1-one, and Nona-3,5-dien-one. 2-Heptanone was found in the pregnant female's urine. The two monoterpenes found included D-Limonene and 1,3,8-p-Menthatriene. Phenols identified included 4-methylphenol, 3-methylphenol, 2,4 dimethylethylphenol, and 2,3,5,6 tetramethylphenol. Esters, acids and benzene derivatives were also common. Unusual compounds included D-Limonene, Napthalene, -xylene, Eicosane, and dihydromethylfuran.

*Non-pregnant females:* The female elephants had ketones as a major compound class, but not the males. All females had 4-Heptanone, 3-Buten-2-one and 2-Buten1-one-1 in their urine, irrespective of breeding condition. 2-Heptanone was also identified in female suckling her calf, while cyclohexanone was identified in the nulliparous female's urine. Nona-3,5-dienone and 3-Hexen-2-one, coumarin and 5,7 Octadien-4-one were putatively identified ketones in adult female urine. The esters, benzoic acid and benzoic acid-2, were also confirmed by NIST in all three females urine, as was Decane-2,-3,-4 and -5.

Unlike previously published research, compounds previously associated with male Asian elephants in musth were here identified in the urine of African elephant adult females. These compounds included cyclohexanone (Nuanedi) and 2-Heptanone (Mussina and Shan). Unlike the pregnant female, urine contained ergosterol, sesquirosefuran, oxepine, pyridine, 1H Pyrrole, 4-carene, cyclohexene, demelverine, coumarin, and octadienone. Both the pregnant and the suckling female had m-cresol and p-cresol in their urine, whereas the nulliparous female did not. Trans–Carveol – an allomone in pine beetles, was found in the suckling female's urine. Other citrus- related terpenoids such as Limonene was noted. A sesquiterpene, Caryophyllene, an attractant in European Oak Bark Beetles (Vrkocová et al. 2000), was identifiend in the suckling female's urine. Unique to her were also the following ketones 3 Hexen-2-one, Coumarin and 5,7 Octadien-4-one.

**Table 4.3:** Volatile organic compounds (>70% NIST match), and the class of chemicals they belong, detected in the urine of female African elephants.

| | Compound | Compound category |
|---|---|---|
| 1 | 1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-ol | Alcohol |
| 2 | 3-Octanol | Alcohol |
| 3 | Ergosterol | Alcohol |
| 4 | Phenylethyl Alcohol | Alcohol |
| 5 | 1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl- | Aldehyde |
| 6 | **2-Propenal, 2-methyl-3-phenyl-** | Aldehyde |
| 7 | **Acetaldehyde**, (3,3-dimethylcyclohexylidene)-, (Z)- | Aldehyde |
| 8 | **Benzaldehyde *** | Aldehyde |
| 9 | 1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl- | Aldehyde |
| 10 | Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl- | Alkane |
| 11 | Cyclohexane, 1-methylene-4-(1-methylethenyl)- | Alkane |
| 12 | Decane, 2-methyl- * | Alkane |
| 13 | Decane, 3-methyl- | Alkane |
| 14 | Decane, 4-methyl- | Alkane |
| 15 | Decane, 5-methyl- | Alkane |
| 16 | Eicosane | Alkane |
| 17 | Pentadecane, 7-methyl- | Alkane |
| 18 | Tridecane | Alkane |
| 19 | Undecane * | Alkane |
| 20 | 1,3,5,7-Cyclooctatetraene | Alkene |
| 21 | 1,6-Dimethylhepta-1,3,5-triene | Alkene |
| 22 | 2-Pentene, 3,4-dimethyl-, (Z)- | Alkene |
| 23 | 4-Carene, (1S,3R,6R)-(-)- | Alkene |
| 24 | 6-Isopropyl-1,4-dimethylnaphthalene | Alkene |
| 25 | Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)- | Alkene |
| 26 | Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl- | Alkene |
| 27 | Bicyclo[4.2.0]octa-1,3,5-triene | Alkene |
| 28 | Cyclohexene, 1-methyl-3-(1-methylethyl)- | Alkene |
| 29 | Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)- | Alkene |
| 30 | Cyclohexene, 3-methyl-6-(1-methylethylidene)- | Alkene |
| 31 | Cyclohexene, 4-methylene-1-(1-methylethyl)- | Alkene |
| 32 | 1-Hexen-3-one, 2,5,5-trimethyl- | Alkyne |
| 33 | 2,4,6-Trimethyl-1,3-phenylenediamine | Amine |
| 34 | Benzenamine, 3-methyl- | Amine |
| 35 | p-Aminotoluene | Amine |
| 36 | (+)-Epi-bicyclosesquiphellandrene | Arene |
| 37 | 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]- | Arene |
| 38 | 2-Methyl-2-bornene | Arene |
| 39 | Ethylbenzene | Arene |
| 40 | **Furan**, 2,3-dihydro-3-methyl- | Arene |
| 41 | Naphthalene *, 1,6-dimethyl-4-(1-methylethyl)- | Arene |
| 42 | Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl- | Arene |
| 43 | o-Xylene | Arene |
| 44 | Oxepine, 2,7-dimethyl- | Aromatic compound |
| 45 | **Benzoic acid * **** | Carboxylic acid |
| 46 | 3-Methyl-2,3-dihydro-benzofuran | Cyclic ether |

| | Compound | Compound category |
|---|---|---|
| 47 | 5-Isopropyl-3,3-dimethyl-2-methylene-2,3-dihydrofuran | Cyclic ether |
| 48 | **Furan, 2-methyl-5-**(1,1,5-trimethyl-5-hexenyl)- | Cyclic ether |
| 49 | Sesquirosefuran | Cyclic ether |
| 50 | Butanoic acid, 2,3-dimethyl-, methyl ester | Ester |
| 51 | Cyclohexanecarboxylic acid, methyl ester | Ester |
| 52 | Hexanethioic acid, S-propyl ester | Ester |
| 53 | Isobutyl isothiocyanate | Ester |
| 54 | **Isobutyric acid**, hexadecyl ester | Ester |
| 55 | Coumarin *, 3,4-dihydro-4,4,7-trimethyl- | Ester |
| 56 | 1,4,7,10,13,16-Hexaoxacyclooctadecane | Ether |
| 57 | 1,4-Benzenediol, mono-tetradecyl ether | Ether |
| 58 | Pentaethylene glycol monododecyl ether | Ether |
| 59 | 2,4-Cycloheptadien-1-one, 2,6,6-trimethyl- | Ketone |
| 60 | **2-Buten-1-one**, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- | Ketone |
| 61 | **2-Heptanone** | Ketone |
| 62 | 2-Nonen-4-one | Ketone |
| 63 | 3-Buten-1-one, 4-[2,6,6-trimethyl-1(or 2)-cyclohexen-1-yl]- | Ketone |
| 64 | 3-Hexen-2-one | Ketone |
| 65 | **4-Heptanone** | Ketone |
| 66 | 4-Hydroxy-3-methylacetophenone | Ketone |
| 67 | 5,7-Octadien-4-one, 2,6-dimethyl-, (E)- | Ketone |
| 68 | **Acetophenone** | Ketone |
| 69 | **Cyclohexanone**, 2-(1-methylethylidene)- | Ketone |
| 70 | Ethanone, 1-(6,6-dimethylbicyclo[3.1.0]hex-2-en-2-yl)- | Ketone |
| 71 | Nona-3,5-dien-2-one | Ketone |
| 72 | Tricyclo[6.3.0.0(1,5)]undecan-10-one, 4-[(2-methoxyethoxy)methoxy]-5,11-dimethyl- | Ketone |
| 73 | 1,3,8-p-Menthatriene | Monoterpene |
| 74 | **Limonene** * | Monoterpene |
| 75 | **Disulfide, dimethyl** | Organosulfur |
| 76 | 3-Methyl-4-isopropylphenol | Phenol |
| 77 | 3-tert-Butyl-4-hydroxyanisole | Phenol |
| 78 | Phenol, 2,3,5,6-tetramethyl- | Phenol |
| 79 | Phenol, 2,4-bis(1,1-dimethylethyl)- | Phenol |
| 80 | Phenol, 2-butyl- | Phenol |
| 81 | **Phenol, 3-methyl-** | Phenol |
| 82 | **Phenol, 4-methyl-** | Phenol |
| 83 | Durohydroquinone | Quinone |
| 84 | Caryophyllene | Sesquiterpene |
| 85 | 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)- | Terpenoid |
| 86 | 1,4-Cyclohexadiene, 3-ethenyl-1,2-dimethyl- | Terpenoid |
| 87 | 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)- | Terpenoid |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. (2002, 2005, 2006, 2012, 2016), * confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

## 4.4 Discussion

This study, whether elephants could discriminate between conspecifics using scent, was partly motivated by the discovery that African elephants were able to discriminate between different humans from skin VOCs (Chapter 2, von Dürckheim et al. 2018). Both male and female African elephants analysed here used olfactory cues in conspecific urine and dung to discriminate between familiar and unfamiliar conspecifics. Since sex was controlled for (donors were the same sex), discriminatory responses indicated olfactory differences between samples based on a combination of variable (age, reproductive state, diet) and fixed (genotype, sex, identity) information. Results support the expectation that elephants would show more interest in the scent of unfamiliar conspecifics, and despite the small sample size, the predictions that urine would encode age and sex appeared valid.

The GC-MS and XCMS results indicate that urinary metabolites in males and females differ significantly, and our results clearly support the behavioural findings. Results suggest that urinary volatiles profiles carry variable (age) as well as fixed (identity, sex) information about the signaller. These results are consistent with similar studies confirming individual differences in urinary profiles in a number of species including wolverines (*Gulo gulo*), marmosets (*Callithrix jacchus*), giant pandas (*Ailuropoda melanoleuca)*, and ferrets (*Mustelo fero*) (Wood et al. 2009, Koyama 2004, Zhang et al. 2005, Yuan et al. 2004). Sex and age are encoded chemically in many vertebrate and mammalian species (Caspers 2011). This research found that sex and age are differentially encoded in olfactory signals in TGS, buccal and genital secretions (see Chapters 5 and 6). Despite the small sample size, MDS scaling plots and iPCA clearly separated into the sexes, and putatively suggested the chemical similarity between the odour profiles of father and son but not between mother and son, raising the possibility that phenotype matching could be an olfactory mechanism underlying parent-offspring recognition and mate choice in African elephants. The chromatograms of the related adult females appeared very similar (Figure 4.5), however it seems that compound concentrations differed among individuals. This has been suggested by prior research on Asian elephant female urine, which changes in concentration prior to ovulation and where steroids 5a-androst-2-en-17-one and -17b-ol concentrations change with ovarian activity and the reproductive cycle (Apps et al. 2015).

The preference tests further indicated that males investigated the urine of unfamiliar conspecifics for longer than the females, while no such sex-related differences were found for investigatory behaviour of dung. The preference for unfamiliar females by males is supported by a similar experiment on African elephant vocalization, which found that males could discriminate between the rumbles of familiar and unfamiliar females, and orientated longer to vocalization of unfamiliar females (Stoeger & Baotic 2017). Behavioural studies on African elephants further support our findings, showing that adult male elephants showed the greatest interest in urine and dung of female conspecifics upon approaching waterholes (Merte et al. 2010), with chemosensory behaviour exhibited by pubescent and adult males towards unfamiliar adult female urine (Loizi et al. 2009). In another endangered mammal, male pandas were most responsive to urine of unfamiliar females (Swaisgood, et al. 1999). Among primates, common marmosets, tufted capuchins (*Cebus paella*) and lemurs (*Lemur catta*) likewise investigated the scent of unfamiliar conspecifics for longer that familiar conspecifics (Henkel et al. 2015). Mole rats (*Cryptomys anselli*) and red squirrel (*Tamiasciurus hudsonicus*) males also indicated preferences for the scent of unfamiliar females (Vache et al. 2001, Heth et al. 2004). Elephant bulls are polygynous, searching and competing for receptive females, while females exercise mate choice. Sexual selection theory suggests that intrasexual competition determines reproductive success (Clutton-Brock 2007) and opportunity costs of inbreeding avoidance and male-male competition can be high. Consequently, the olfactory ability of elephant bulls to successfully identify receptive and unrelated females from urine and dung left along pathways and at waterholes is vital, where bulls can ill afford olfactory mistakes given their declining body condition during musth and the rarity of finding a receptive mating partner. In Amboseli in Kenya, long term studies have shown that African elephant males prefer mating with non-kin they are not familiar with, however the mechanism underlying this kin discrimination is not fully understood (Archie et al. 2007), but phenotype matching has been suggested. Sexual dimorphism in olfactory behaviour among African elephants has been suggested to be a product of signal function, where males actively seek out receptive mates, while females monitor other female's reproductive and social status within the herd. Elephant females appear

to recognize their kin, and they are able to monitor up to 30 family members from urine, including absent kin (Bates et al. 2008).

Given that elephants spent more time investigating urine than dung in this study, urinary metabolites were characterised. For an overview of reproductive signalling in elephant dung in *Elephas maximus*, please refer to Ghosal, Seshagiri and Sukumar (2012). Previous research has suggested that urine is important to sexual signalling in elephant as females monitor conspecific oestrus and musth from urine, while it is suggested that calves can recognize their mothers from urine (Rasmussen 1995). Male African elephants can discriminate between follicular and luteal phases of oestrus in urine (Bagley et al. 2006). Social signalling cues in elephant secretions are less well understood, however African elephant females urinate during ritualized greeting ceremonies, which are presumed to affirm social relationships among fission-fusion mammals (East 1993). Our results that elephant urine encoded identity and age supports the concept of a social signal, but larger studies with statistically significant samples sizes are required to expand on these results, and to test a signal for dominance rank among adult females.

Urinary chemical composition and diversity of three adult African elephant females urine, in different breeding conditions – namely: pregnant, suckling and nulliparous, and compared with the urine of two adult males, one breeding male and one treated with GNRH, was putatively described using NIST probability matches of >70%. Elephant urine had richer compounds than TGS, buccal and genital secretions, with significantly more chemical compounds in the signature mix (Tables 4.3 and 4.4). Elephant urine contained more ether compounds and fewer aldehydes than temporal gland secretions, buccal and genital secretions from the wild-sampled population (Chapters 5 and 6). Urine has a vast array of complex chemicals that encode information on individual variation, as it contains both variable and fixed factors, in a variety of species. Although compounds of mammalian as well as non-mammalian, yet natural origin (Charpentier et al. 2012), were found in elephant urine (3-octanol, Pentadecanoic acid, 9-octadecanoic acid (Z) methyl-ester, .alpha.-phellandrene, 1,3,8 menthatriene, decahexaundecane and octanoic acid, 2-octanone, 2-undecanone and limonene), the focus on

origin of chemicals must be exercised with caution as not all semiochemicals of significance are animal metabolites (Apps, Kramer & Waldon 2015).

Previous research on semiochemicals in African and Asian elephants used GC-MS, and found differences in volatile ketones between musth and non-musth urine in bulls (Rasmussen & Wittemyer 2002). In this study, compounds of interest were noted that either confirm or challenge previous findings in elephants, in order to assist with compound selection for future verification and bioassays. Volatile ketones in male urine were identified, although these did not predominate as a compound class in the adult males. Ketones included cyclohexanone, 3-buten-2-one, 2-buten-1-one and 4-heptanone. However, the male calf shared many of the ketones, phenols and alcohols listed in Goodwin et al. (2012) – a study of African elephant adult male musth and non-musth urine. These compounds included 2-Heptanone, 2-Octanone, 2-Decanone, acetophenone, 2-decanol, 2-undecanol and 4-methylphenol. Noteworthy was the high number of alcohols in the GNRH male, the high number of arenes and aromatic compounds in the breeding male and the significant number of phenols in the male calf. Fatty acids, alkan-2-ols, and alkan-2-ones previously identified in African elephant males were confirmed here (Goodwin et al. 2016).

Goodwin et al. (2005, 2006) identified a bark beetle pheromone frontalin and brevicomins in ovulatory urine in African elephant females, as well as aphid alarm pheromones (E,E)- α-and ß-farnesene). Although our study tentatively identified exo-brevicomin in the breeding male, we did not identify frontalin nor farnesenes in the female elephants, which are difficult to detect by SPME (Goodwin et al. 2006). Interesting was the presence of a terpenoid – y-terpinene (or gamma terpinene) in all elephants except the pregnant female. This compound is a confirmed pheromone in bark beetles (*Scolytinae spp.*) and European rabbits (*Oryctolagus cuniculus*), and may require further investigation given that the biosynthesis of brevicomins and frontalins via precursors proceed similarly in beetles and African elephants (Goodwin et al. 2006).

Phenols identified in the urine headspace of Asian pregnant females included 3-ethylphenol and 2-ethyl 4,5 dimethylphenol (Rasmussen & Krishnamurthy 2001). Mussina's urine contained 3 and 4 methyl phenol, 2,4-bis(1,1-dimethylethyl)-phenol, and 2,3,5,6 –tertramethylphenol. Further compounds that the two pregnant females of both species shared in common included

acetophenone and 4-heptanone, but as the latter was found in all elephants in this study (including males), therefore it cannot solely be associated with breeding condition although a larger sample would be required to confirm or rebuke this association.

This study confirmed Goodwin et al.'s (2012) finding of 4-methylphenol (p-cresol) in male and female elephants. P-cresol and benzoic acid, previously identified pheromones in African elephants (Wheeler 1982, Goodwin et al. 1999), were also identified in this study. Unlike previously published research, we found compounds associated with urine of Asian bulls in musth in the urine of African elephant adult females - if the presence of 2-heptanone and cyclohexanone is verified in female elephants, the functional role of these two compounds may need to be revaluated in the scientific literature for both Asian and African elephants, in particular with regards to male-male, male-female, and possibly female-female reproductive signalling. Specifically, novel compounds such as y-terpinene, caryllophene and Trans-carveol identified by NIST should be of interest given that bark beetle and aphid pheromones have been identified in elephant semiochemicals (Goodwin et al. 2006).

Apps et al. (2015) and Drea et al. (2019) have highlighted the importance of acetophenone, 4-heptanone, 2-heptanone, and 2-hexanone, with the latter study suggesting that these compounds constitute possible ancestral chemical urine markers in Strepsirrhines. The former work argues that olfactory signals are not necessarily species specific, with 2-heptanone found to delay puberty in mice, 4-ethyl-phenol signalling territory in beavers, and acetophenone stimulating overmarking. Differences between urine signal and those of TGS, buccal and genital secretions are further explored in the concluding chapter (Chapter 7), however previous work argues that urine due to its complexity, and its high number of semiochemicals, reliably encodes for individual identity (DelBarco-Trillo et al. 2011), and that consistent with gradual signal evolution, the profiles of highly related individuals share more similar chemical profiles. In Africa, elephants travel great distances at night in open Transfrontier Conservation Areas (TFCAs), retreating to parks and refuges during the day (von Dürckheim 2011). It is likely that urine and dung deposited on pathways and at waterholes function as scent marks for intra-specific social as well as sexual signals, and that high volatility in urine may underlie detectability (Drea et al.

2019). The fact that urine contains high molecular weight compounds and fixatives that would encourage the slow release of volatile compounds over time (Apps et al. 2015) is significant.

## 4.5 Conclusion

Although this study design did not consider daily/monthly metabolic fluctuations resulting from reproductive status, diet or health, we found that urine encoded fixed information (gender and individual identity). A large number of substances of varying volatility in male and female African elephant urine were captured using the National Institute of Standards and Technology (NIST) database. According to the Pherobase database, many of these substances are used in the chemical communication system in a variety of taxa, from beetles to bontebok. Some substances are confirmed pheromones across a variety of taxa, however to be confirmed a pheromone in African elephants, notable compounds of interest must be selected, verified by internal standards and tested by conducting behavioural bioassays in the field. Our sampling is likely to be incomplete, however our analyses revealed potentially interesting patterns and compounds that warrant further investigation. This research expands on the previous literature on African elephant urinary semiochemicals, and can lead to collaborations with academic institutions and sanctuaries.

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# Chapter 5: Characterizing the chemical composition of African elephant temporal gland secretions (TGS) using Solid Phase Microextraction (SPME) and Gas Chromatography-Mass Spectrometry (GC_MS)

*"Elephants are contenders for rank among those species possessing Machiavellian intelligence, in every respect. The formation of long-term relationships is the very essence of elephant society and a network of allies, who are usually though not always genetically related, defines the elephant family and bond group."*
*Joyce Poole and Cynthia Moss (1989)*

**Abstract:**

Elephants are considered macrosmatic mammals and the significance of olfaction to elephant social behaviour is well documented. Semiochemical research to date has, however, has focused mainly on captive male Asian elephants *(Elephas maximus*) and reproductive signalling between the sexes. African elephant (*Loxodonta africana*) semiochemicals remain poorly understood, and little empirical data exist on the role of chemical signatures in social signalling and conspecific recognition in the species. The temporal gland is unique to elephants and its secretions appear to serve reproductive and social functions. In Asian elephants, temporal gland secretions (TGS) signal dominance and musth in bulls. In African elephants, the composition and function of TGS for reproductive and social signalling is less well understood. Unlike their Asian counterparts, African elephant females secrete TGS frequently. Female African elephants are socially complex, and live in highly sophisticated fission-fusion systems, which are flexible and occur at multiple tiers of social organization. In this study, TGS was sampled from 106 elephants in 15 herds during a translocation event, and GC_MS, genetic analyses and an array of statistical methods used to determine whether TGS odour encodes sex, age, relatedness and identity in African elephants. TGS encoded individual identity, age and possibly sex in African elephants. No correlation between genetic relatedness and TGS odour was found at the population, herd or individual level. However "herd" was a significant factor in explaining chemical differences between groups, and with the presence of short chain fatty acids of low molecular weights in TGS, gives rise to the possibility that group odour may be a result of bacteria. Next, TGS in 40 wild adult females was characterised for the first time. A total of 165 compounds were identified. Aldehydes, ketones, carboxylic acids and aromatic compounds predominated as compound categories. Compounds associated with male elephants were identified in the African elephant adult females. Results suggest that African elephant TGS carries an olfactory signal for individual identity of bacterial origin that is likely to be beneficial for bond group cohesion within their fission-fusion society, and has management implications for elephant reintroductions.

**Key words**: African elephant, GC_MS, olfaction, relatedness, semiochemicals, temporal gland secretion

## 5.1 Introduction

Chemical communication in mammals is linked to reproduction, mate choice and attraction, territoriality, parental care, kin discrimination and disease transmission, all of which inform population dynamics and structure. Olfactory signals encode fixed and variable information such as age, rank, and sex (Setchell et al. 2010, Marneweck et al. 2017), as well as genetic differences between species and subspecies (Heth et al. 2004, Heth et al. 2003, Busquet & Baudoin 2005). At a fine scale, research has revealed that chemical signals encode information about genetic quality and relatedness in fur seals *(Arctocephalus gazella*) (Stoffel et al. 2015) and lemurs (*Lemur catta*) (Charpentier et al. 2008, Boulet et al. 2009). Recently Kean et al. (2017) found that otters (*Lutra lutra*) possess chemical signals for sex and biogeography-specific odours. Chemical signals have been significantly correlated with individual identity in hyenas (*Crocuta crocuta*) (Burgener 2009) and raccoons (*Procyon lotor*) (Kent & Tang-Martinez 2014). Chemical expression of genetic relatedness in an individual has been proposed to promote outbreeding, facilitate nepotism and function in phenotype matching, kin discrimination (Boulet et al. 2009) and mate choice and a number of studies have explored the correlation between olfactory phenotype and MHC genotype (Setchell et al. 2010, Wedekind et al.1995, Penn & Potts 1998).

Mammals that live in socially complex systems require an effective means of communication in order to maintain social cohesion, and a number of acoustic, olfactory and visual signals have been described (Wyatt 2003). Within a group context, individuals need to discriminate between members of their group and foreign conspecifics in order to avoid inbreeding and to identify recipients for reciprocal behaviour. Group odour has been suggested as a mechanism whereby conspecifics can distinguish group from non-group members. Such olfactory group/herd/clan/colony identity signals have been described in a number of mammals such as beavers (*Castor canadensis*) (Sun & Muller-Schwarze 1998), bats (*Eptesicus fuscus*) (Bloss et al. 2002), naked mole rats ( *Heterocephalus glaber*) (O'Riain & Jarvis 1997), fur seals (Stoffel et al. 2015), hyenas (Burgener 2008), chimpanzees (*Pan troglodytes*) (Henkel & Setchell 2018) and rhesus macaques (*Macaca mulatta*) (Henkel et al. 2015).

Elephants are an important group of mammals in the study of olfaction as they use chemicals to communicate (Schulte et al. 2007). Elephants emit chemical signals via their temporal glands, urine, faeces, breath, saliva, interdigital glands, genitalia and body surfaces, and detect both self and non-self signals via their highly sophisticated olfactory, and vomeronasal systems (Jachowksi 2011, Sukumar 2003). These chemical signals influence elephant behaviour, social interactions and reproduction (Greenwood et al. 2005, Slotow et al. 2000). Elephant reliance on olfaction is also reflected in its genome, which contains ~2000 functional olfactory receptor genes and >2200 pseudogenes, the highest number of olfactory receptor genes of any mammal (Niimura et al. 2014). Unique to elephants is the temporal gland, a modified apocrine structure imbedded in the subcutaneous tissue on each side of the head between the eye and the ear. Aqueous secretions from the temporal gland have a pungent and distinct odour. It is a confirmed important chemosignalling source in elephant males (Rasmussen 1999, Rasmussen & Schulte 1998, 1999). Secretions from the temporal gland contain lipids, glycoproteins, proteins, polysaccharides and volatiles from both apocrine origin and blood (Rasmussen & Krishnamurthy 2000). Chemical compounds in the TGS vary between individuals based on age and reproductive state. In Asian elephants, TGS are associated with musth in bulls, a period of elevated testosterone and heightened aggression associated with reproductive status and dominance. Asian adult females secrete TGS infrequently, usually during pregnancy and birth. A striking difference between the Asian and African savannah elephant is that in the latter species, both sexes secrete TGS. TGS is significantly correlated with age, with adult females more likely to secrete TGS than males (Kioko et al. 2017). Unlike Asian elephant females, African females secrete TGS daily, typically when excited, distressed or during social interactions. African elephants, like black-tailed deer (*Odocoileus columbianus*), rub their forehead/temporal orifices on branches and twigs. TGS may function as olfactory signposts for long-distance communication (Muller-Schwarze 1972, Buss et al. 1976, Langbauer 2000). When herds comprised of related females and their offspring meet one another, elephants perform a ritualised greeting ceremony in which elephants press their bodies against one another, trumpet, rumble, entwine their trunks, click tusks, secrete TGS, urine and dung profusely, while fanning their uplifted ears and spinning their bodies (Moss 1988). The

fanning of ears can be likened to the fanning of wings, which is a strategy adopted by Lepidoptera to disperse pheromones (Kindl et al. 2011). Although no empirical studies have explained the function and the content of these chemical emissions in African elephant females, it is likely that these odour signatures underlie recognition in elephants and possibly promote bond group cohesion (Wyatt 2003, Buss et al. 1976, Rasmussen 2002).

Most vertebrates recognise close relatives (kin recognition) to avoid mating with them or to establish cooperative partnerships with relatives (kin discrimination). In unperturbed populations, kinship in African elephants is a primary determinant of elephant social relationship both within and across core social groups, which fission and fuse in response to resource availability (Archie et al. 2006). In depredated populations marked by poaching, evolutionary drivers for sociality are sufficiently powerful to maintain group structure and linear dominance hierarchies among unrelated conspecifics within a herd (Wittemyer et al. 2009). The maintenance of familial relationships across an elephant population of several hundred conspecifics necessitates the ability to identify and locate many individuals, yet the olfactory mechanism to discern familiar and related conspecifics remains elusive, although auditory mechanisms have been described (McComb et al. 2003). Olfactory discrimination of familial conspecifics and kin is typically explained by associative learning. Bates et al. (2008) have shown that African elephants can recognize up to 17 females and possibly up to 30 family members from cues present in the urine–earth mix, and that they keep track of the location of these individuals in relation to themselves. However, research suggests that African elephants discriminate paternal kin from non-kin despite their polygynous mating system (Archie et al. 2007). This suggests that males may be using phenotype matching to identify and avoid mating with unfamiliar kin. Phenotype matching has been demonstrated in other vertebrates, and the use of olfactory cues explored (Brown & Eklund 1994, Zelano & Edwards 2002). In elephants, research suggests that the signal may be olfactory, however it is unknown whether olfactory signals are closely correlated enough with relatedness to be sensitive cues for phenotype matching, and which scent gland may produce the signal. In other mammals, semiochemical analyses on family members discovered convergent semiochemical profiles among colonially living, related beavers (Sun & Muller Schwarze 1998b), while a similarity of chemical profiles

without genetic correlation was revealed in maternal lines in colonially-living bats (Safi & Kerth 2003).

TGS have been comprehensively researched in captive Asian elephant bulls (Rasmussen 2001, Rasmussen & Schulte 1998, Rasmussen & Perrin 1999, Rasmussen & Greenwood 2003, Schulte & Rasmussen 1999) and to a lesser extent in captive African elephant bulls (Rasmussen, Riddle & Krishnamurthy 2002, Rasmussen, Hall-Martin & Hess 1996, Goodwin et al. 2002; 1999, Schulte et al. 2006, Wheeler et al. 1982), with African elephant samples generally being limited in number, and from a captive environment. In both species, a variety of substances and volatiles have been identified, including lipids, glycoproteins, polysaccharides, volatiles, and steroid hormones (Rasmussen & Krishnamurthy 2000). In Asian elephants, TGS is associated with reproductive and dominance signalling in males, containing serum testosterone and dihydrotestosterone at elevated levels, while immature elephant males emit sweet-smelling, honey-like TGS to avoid conflict with adult males (Rasmussen, Riddle & Krishnamurthy 2002). Females can distinguish males in musth using olfactory cues from compounds (cyclohexanone) in TGS of Asian elephant in musth (Rasmussen & Perrin 1999), while differential behavioural responses of Asian elephant males and females to frontalin – a bark beetle pheromone and a component in Asian elephant TGS, in older musth males, was recorded (Rasmussen & Schulte 1998, Rasmussen, Lazar & Greenwood 2003). Dry samples of the temporal gland in two captive Asian elephants revealed the presence of 2-butanone, as well as aldehydes, ketones, butanal, acetone and isoprene and acetic acid, and numerous aldehydes (pentanal, hexanal, decenal, octanal and nonanal) in the post-musth phase (Rasmussen 2001). Semiochemical research in African elephants has received less attention than its Asian counterparts. Research to date has revealed phenols, sesquiterpenes, sesquiterpenoids and terpenoids in the temporal gland secretions in African elephants (Wheeler et al. 1982, Goodwin et al. 2002, 1999).

Given the difficulty of procuring samples from free-ranging individuals, the paucity of scientific data on African elephant TGS is not surprising. A recent behavioural study suggested that the secretion and timing of TGS is associated with elephant specific variables such as sex, age, and body condition, and is more frequently secreted by adult females (Kioko et al. 2017).

However, to date no study has examined the volatile organic compounds (VOCs) release in TGS in elephant adult females, nor the information that is conveyed, in a wild population of African elephants. To address this, we examine the long-held assumptions that TGS may encode chemical signals for age, sex, relatedness, and identity in African elephants by sampling TGS from 106 (of 113) elephants during a translocation event. We further describe the chemical composition of TGS in general, and more specifically in adult females, and compare key compounds with published libraries such as NIST. This study explores whether a) odour profiles differ between age categories and sex and b) whether elephants share an olfactory signal encoding genetic relatedness and herd membership.

## 5.2 Methods

**5.2.1 Study site and field methods**: Following the reintroduction of 213 African elephants into Majete Wildlife Reserve (MWR) in 2010, the elephant population had increased to 390 individuals (Forrer 2016). To relieve population pressure in MWR, management authorities of MWR relocated elephants to Nkhotakota Wildlife Reserve (more information can be found at www.500elephants.org) in 2017. Elephants were translocated in family units by the Department of National Parks and Wildlife (DNPW) in Malawi in partnership with African Parks (AP) PTY Ltd. Translocation of elephants (sighting, selection, immobilization, and transport) and veterinary services were conducted by translocation specialists Conservation Solutions (CS) under special permits. Permission was granted to Stellenbosch University by DNPW, AP and CS to sample chemical swabs and to, under supervision, collect blood samples for DNA analysis from 113 wild African elephants darted in Majete Wildlife Reserve prior to translocation to Nkhokotakota Wildlife Reserve. Majete Wildlife Reserve (S15° 54’26.6”; E034°44’24.3”) is located at the southern tip of the Great Rift Valley, in the Lower Shire Valley region of southern Malawi and covers 700km$^2$.

**5.2.2.Ethics Statement:** Research protocols were cleared by USAMRMC (US Army Medical Research and Materiel Command) Animal Care and Use Review Office (ACURO) and by Stellenbosch University IACUC (The Institutional Animal Care and Use Committee) - Ethics Approval Protocol Number: SU-ACUM15-00002, USAMRMC proposal 65978-ST-ITC, Award

W911NF-14-1-0596. Samples were collected in accordance with the Convention of International Trade in Endangered Species of Fauna and Flora (CITES Permit # Malawi 171383, CITES Permit # South Africa 000054), and retained under permits issued by the Department of Environmental Affairs (DEA Permit # 07901).

**5.2.3 Chemical analyses:** Temporal swabs were obtained from 106 African elephants immobilised during a translocation event, by rubbing the temporal gland of each elephant using 3 x sterile COPAN cotton wool swabs immediately after darting. Not every elephant could be sampled for TGS as infants and calves do not secrete TGS. Samples were stored at -20°C in 20ml clear precision screw-thread glass vials. GC-MS analyses occurred three months after sampling, and samples were transferred into a 20mL SPME headspace vial and sealed with a polytetrafluoroethylene (PTFE, Teflon®)/silicone septa and steel cap. As an internal standard, 50µL of Anisole d8 was added. Vials were equilibrated at 30°C for 5min using a CombiPAL solid-phase microextraction (SPME) autosampler (CTC, Switzerland). A conditioned (conditioned by heating in a gas chromatograph injection port at 20°C for 60min) fibre coated with a 50/30µmm thickness of divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) was inserted into the headspace above the sample and held for 30min (with agitation). The fibre was consequently withdrawn into the needle by the autosampler and inserted into the injection port of a 6890N gas chromatograph (GC) (Agilent Technologies, Palo Alto, CA, USA) coupled to a mass spectrometer detector 5975B (Agilent Technologies). The SPME fibre was desorbed and held in the injection port (250°C) for 10min. The fiber was then inserted into a fibre conditioning station for 10mins between samples for cleaning to prevent cross-contamination. The injection port was operated in pulsed splitless mode. Volatile compounds were separated using a polar ZB-Wax capillary column (30m, 0.25mm i.d. 0.25µm film thickness). The oven temperature was initially held at 40°C for 5min and increased to 240°C at 5°C/min (held for 3min). The total run time was 48min. Helium was used as the carrier gas with a constant flow rate of 1.9mL/min. The transfer line was maintained at 280°C. The mass spectra were obtained using a mass selective detector working in electronic impact at 70 eV, operated in full scan mode (35-450m/z) with both the ion source and quadrupole temperatures maintained at 240°C and 150°C, respectively. Semiochemicals that had consistent retention times and accounted for

> 0.05% of the area of the total chromatogram were retained. For pre-processing of GC_MS data (peak alignment and normalisation), a custom-written R script (GCAlignR) written by Ottensman et al. (2018) and Stoffel et al. (2015) was used that compensates for minor shifts in retention times among chromatograms by maximizing the number of shared components between samples through minor shifts in the retention time.

**5.2.4 Genetic analysis:** Blood was taken from the auricular vein of the elephants by a wildlife veterinarian using 5 x 4ml sterile EDTA vacutainer tubes. Samples were stored on ice between darting events, and frozen within an hour of extraction. Genomic DNA was extracted one month after the translocation from whole blood of 106 individuals using the Prepfiler Automated Forensic DNA extraction kit (Thermo Scientific) and purified on the Kingfisher Flex Purification System (Thermo Scientific). Individuals were genotyped at 18 microsatellite loci using two multiplex panels that comprise previously reported loci from Archie et al. (2003), Comstock et al. (2000) and Eggert et al. (2003). For quality control 11 individuals were re-extracted and re-genotyped across the full panel. For each multiplex panel PCRs and electrophoresis were performed on 10µl reactions using the KAPA2G™ Fast Multiplex PCR Kit (Kapa Biosystems). Amplification PCRs were performed on a GeneAmp PCR System 9800 as follows: 95°C for 3min; 30 cycles of 95°C for 15sec, 60°C for 15sec and 72°C for 30sec; and a final amplification at 72°C for 10min. Electrophoresis was performed on a 3500x Genetic Analyzer (Thermo Scientific) and the resulting data were analysed in STRand (Toonen & Hughes 2001) using the GeneScan™ 500 LIZ® size standard (Thermo Scientific). To estimate pairwise genetic relatedness between individuals, the software Identix (Belkhir et al. 2002) was used to calculate Queller and Goodnight's RQG (Queller & Goodnight 1989) for each dyad across the data set.

**5.2.5 Statistical Analysis Framework:** For statistical analyses of the semiochemical data, peaks that were present in only one sample were excluded, which left a total of 165 retained substances for the complete dataset. To estimate the maximum number of substances within the samples and to test for completeness of sampling, the maximum number of substances using the Michaelis-Menten function with a permutation procedure of 9999 iterations was applied. Analyses were conducted on the relative proportion of each peak in the chromatogram to the overall area of an individual profile. The data were then visualized and statistically

analysed for patterns of chemical similarity in relation to sample population, herds, adult sister pairs and relatedness. Computer code for R (GCAlign) was adapted from Ottensman et al. (2018), a package specifically developed for ecological and evolutionary research, evaluating similarity patterns across multiple and variable biological samples.

**5.2.5.1 Overall patterns of chemical similarity:** Chemical profiles were visualized using multidimensional (MDS) scaling ordination and nonmetric multidimensional scaling (nMDS) based on Bray-Curtis Similarity Values calculated from the log(x+1) transformed data. Each point in the 2-dimensional MDS plot represents an individual elephant swab, with clumped points representing individuals with greater chemical similarities. MDS has been successfully used in other studies to visualize chemographic data in mammals (Morelli et al. 2013). Differences between a priori defined groups (herds and -sister dyads) were then analysed using ANOSIM, a non-parametric test for group differences, using 999 iterations of the dataset (Oksanen et al. 2015). ANOSIM is a permutation test that tests for significant differences between groups without the need for assumptions on homoschedacity and data distribution. These analyses were implemented in R using the vegan package (Dixon2003).

**5.2.5.2 Partial Mantel Tests**: Using the full complement of swabs, we performed a correlation of relatedness against chemical similarity using the genetic relatedness matrix based on 18 loci as the response variable and fitted pairwise Bray-Curtis Similarity Matrices as predictor variables using a Partial Mantel test. Separate models were constructed for a priori defined groups (herds, sister-dyads) using 10000 permutations of the dataset. Finally, Spearman Rank Correlation (Mantel's r) and two-tailed P-value for the association between relatedness and chemical similarity were computed.

**5.2.5.3 Identification of chemicals:** In order to assess the contribution of specific chemicals to the similarity within and between groups, we used a similarity percentages process (SIMPER). All Bray-Curtis similarities within a group are decomposed into percentage contributions per compound, listing the compounds in decreasing order of importance. Individual herds were specified as "groups".

**5.2.5.4 Identification of compound type:** The type of compound represented by each peak was identified by filtering compounds by quality. Compounds were retained that had a

probability match of >70%. Identification of putative substances was based on retention times, and by comparing mass spectra with the best match of the library of the National Institute of Standards and Technology (Gaithersburg, MD, USA). Compounds were categorized into compound categories. Exact identification of each compound, through injection of commercial standards, was considered unimportant for the present study (as in Scordato et al. 2007). Specifically, chemical compounds of adult female TGS is characterised.

## 5.3 Results:

**5.3.1 Genetic and chemical data:** DNA was sampled from 113 elephants in 15 herds during the translocation event (Table S5.1). Subjects included 40 adult females, one adult male, nine subadults, 32 juveniles, 19 calves and 12 infants (Table 5.1). Park management targeted family units for translocation, hence the absence of mature males. DNA samples were genotyped at 18 highly polymorphic microsatellite loci (~ no alleles /locus: 5.778, ~ proportion of loci typed: 0.9985 and ~E heterozygosity: 0.6491). None deviated significantly from Hardy–Weinberg equilibrium (HWE), and were therefore retained for subsequent analyses. Pairwise estimators for relatedness (Queller & Goodnight 1989) followed a normal distribution (Figure 5.1). In the translocated population, herds showed mean relatedness coefficients within the limits of their expected distribution (Figure 5.2), ranging from a minimum QG of -0.46 to a maximum of 0.7 within the same herd, and with much less variation (min 0.16 – max 0.51) in an intact elephant core unit (Herd L, matriarch and three female offspring). It is important to note that due to the stressful impact of the translocation process on the elephants (disturbance from helicopter and immobilization), herd membership as noted during the translocation may not reflect original herds. For example, one adult male (age 17 years) joined an unrelated herd during one capture event, while some larger herds separated into basic core units during the helicopter pursuit.

**Table 5.1:** Age /sex classes of sampled elephants from the translocated population (after Moss et al. 2011).

| Age category | | Age class | Males | Females | Total per age class |
|---|---|---|---|---|---|
| Infants | I | 0-1 | 7 | 5 | 12 |
| Calves | II | 2-4 | 14 | 5 | 19 |
| Juveniles | III | 5-9 | 15 | 17 | 32 |
| Sub-adults | IV | 10-14 | 0 | 9 | 9 |
| Adults | V | 15+ | 1 | 40 | 41 |
| **Total** | | | **37** | **76** | **113** |

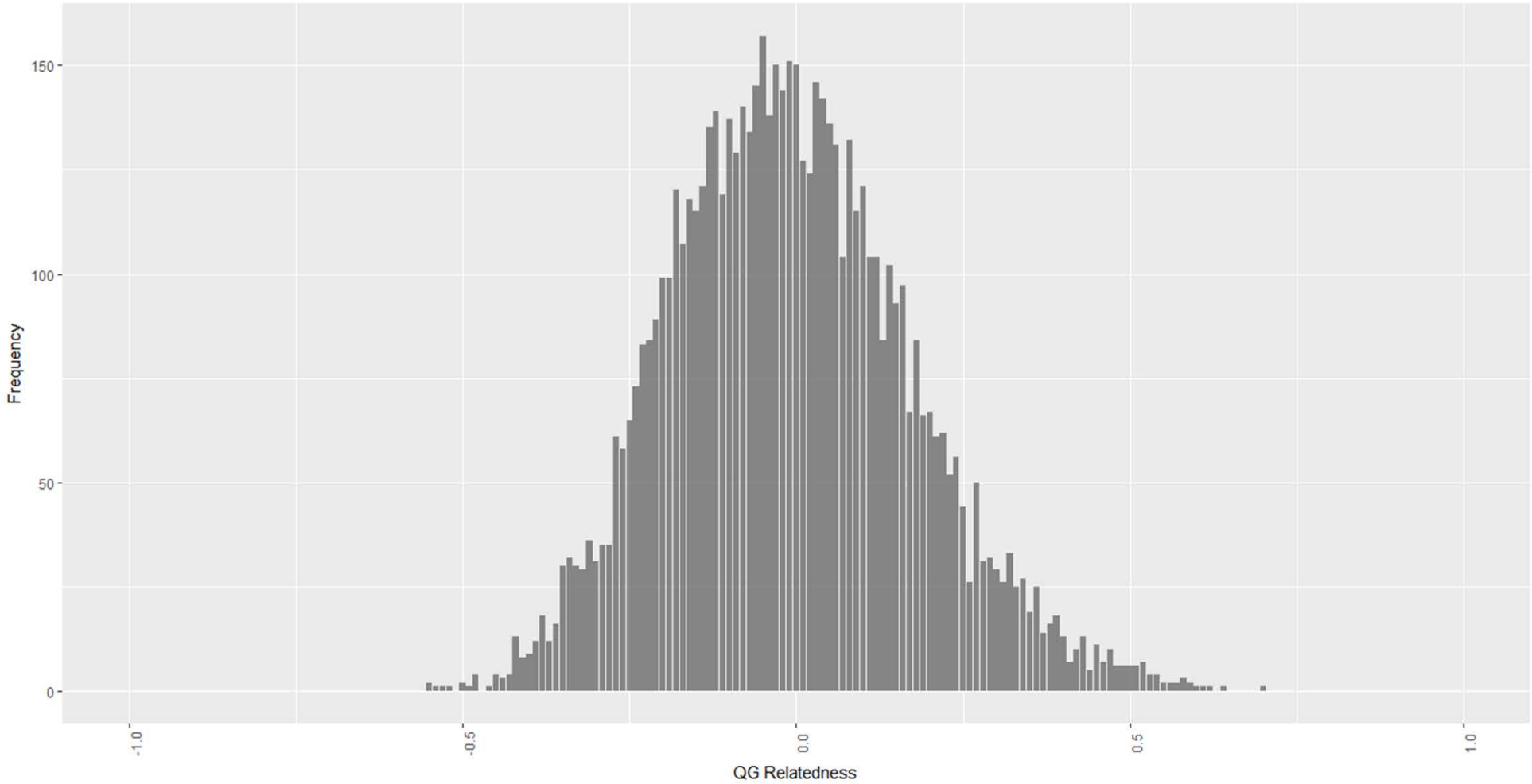


**Figure 5.1:** Frequency data for Queller & Goodnight Pairwise Relatedness values (*r*) followed a normal distribution.

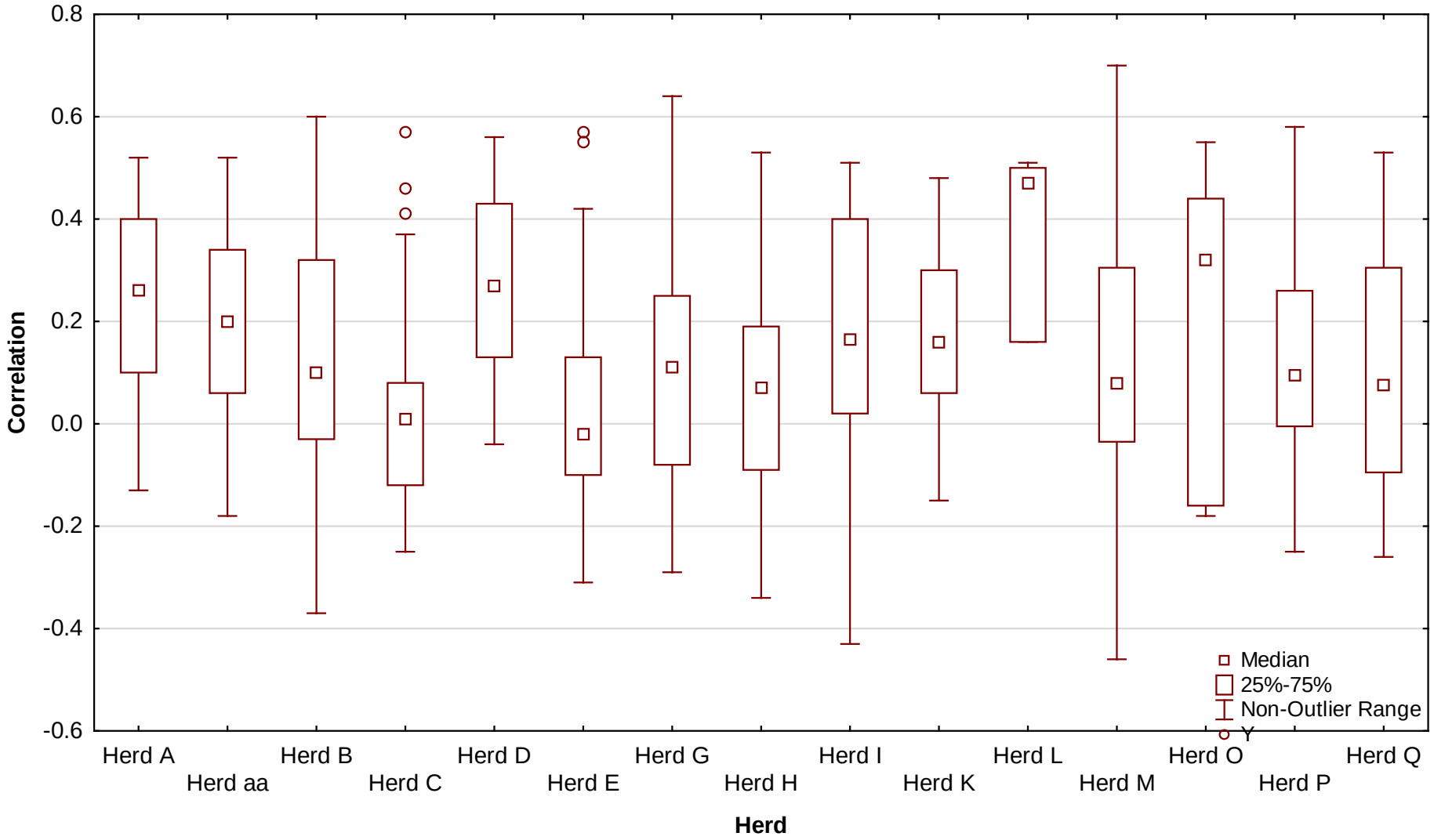


**Figure 5.2:** Box and whisker plot of pairwise relatedness estimates (*r*) of mean relatedness per herd.

**5.3.2 GC AlignR Alignment results**: Temporal samples were collected for analysis from 106 elephants. Seven substances were present in blanks. The corresponding peaks as well as the blanks were removed from the data. Sixty-two substances were present in just one single sample and were removed and one hundred and sixty-nine substances were retained after all filtering steps. The removal of substances from blanks or those present in only one sample reduced the number of peaks present in each sample significantly. For most samples, only minor if any shifts, were required (Figure 5.3). Lastly, the retention times of homologous peaks in the aligned dataset were left-skewed, suggesting that the variation among most substances was less than 0.05 minutes (Ottensman et al. 2018).

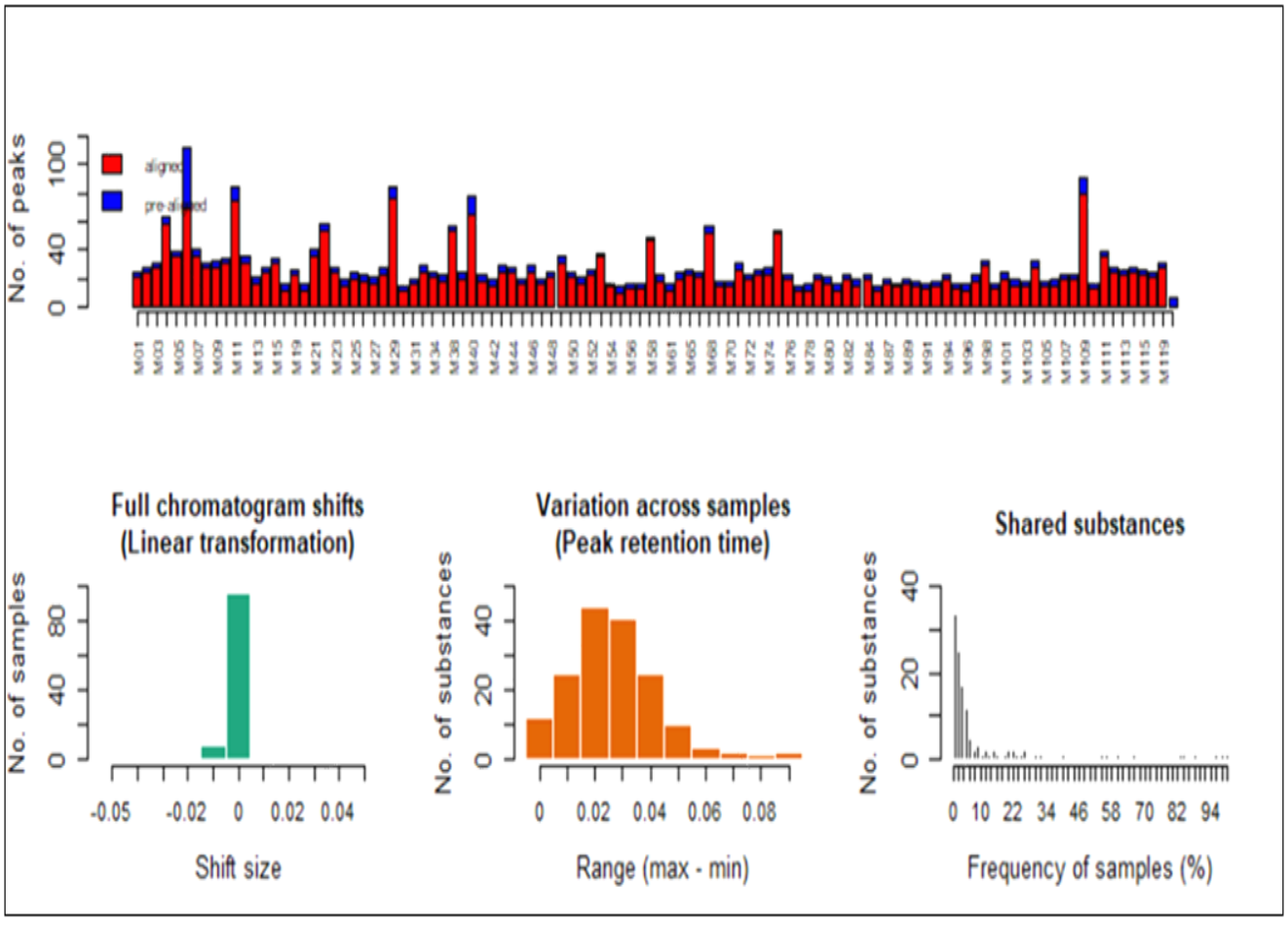


**Figure 5.3:** The first histogram shows the number of peaks per sample before and after alignment. The histogram bottom left shows the full chromatogram shifts (the first step in the algorithm). The bottom middle shows how much peaks vary around the means across samples. The histogram bottom right shows how many peaks are shared across samples.

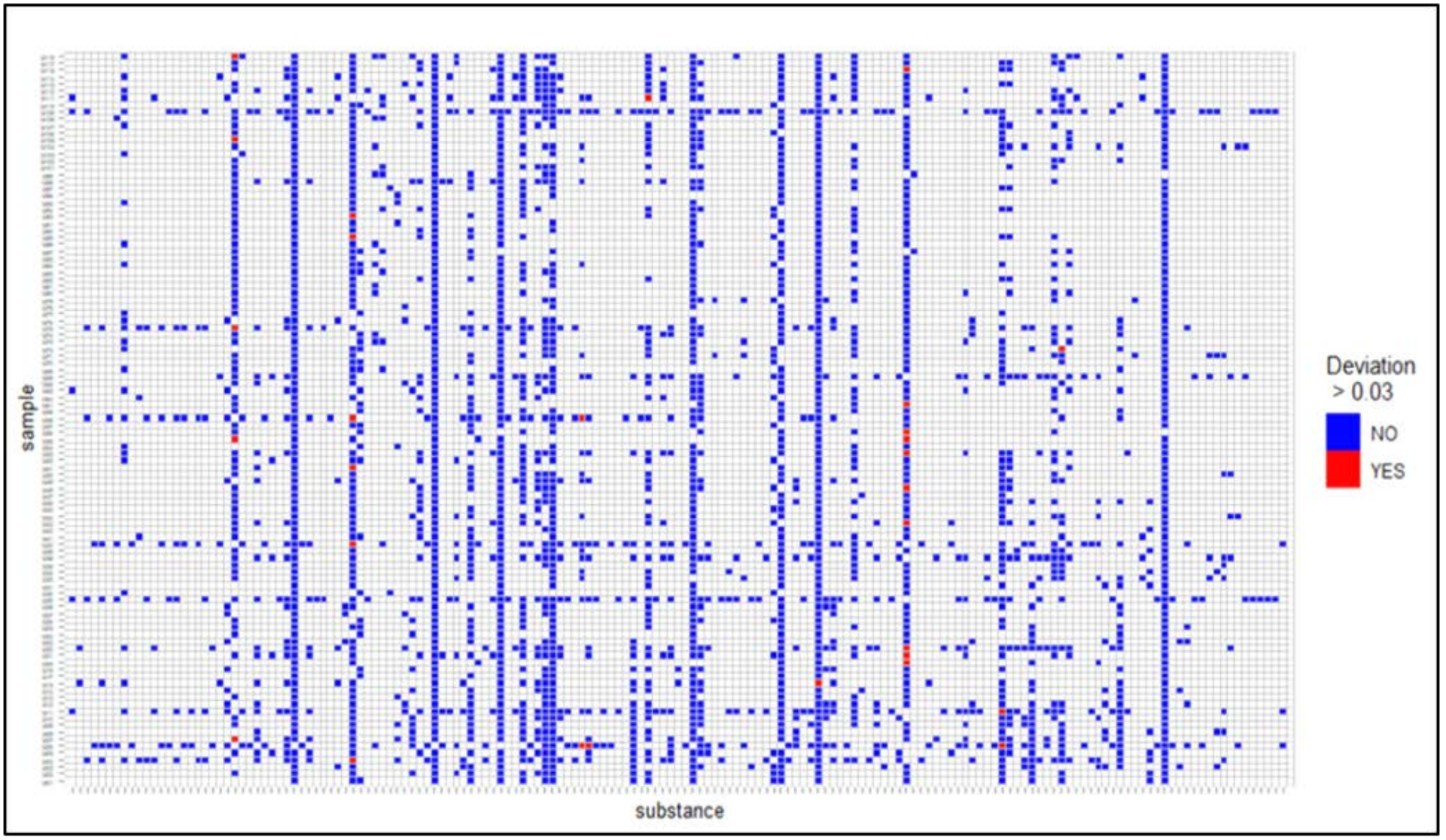


**Figure 5.4:** Presence/absence matrix of compounds (n=169) contained in TGS for African elephants (n=106).

**5.3.4 Herd differences:** Multivariate statistical analysis of the relative proportions of each substance isolated from the 15 herds suggested significant differences in chemical profiles between elephant herds (ANOSIM, global $r^2$ =0.2, $p < 0.0019$) and between elephant ages (ANOSIM, global $r^2 = 0.07$, $p< 0.031$). A low significant effect could be found for sex (ANOSIM, global $r^2 = 1.61$, $p<0.07$).

To further explore the significance of herd as a factor in this translocated population, 15 herds were grouped into three factors of median pairwise relatedness estimate *r*: i) highly related, ii) unrelated, and iii) low relatedness (Table 5.2). For the purposes of this chapter, QG values above 0.2 were considered “kin” (Archie et al. 2007). It was assessed whether individuals from herds with high r are more chemically similar to each other than individuals from herds with low genetic r.

**Table 5.2**: Three categories of median *r* were calculated and tested for significant differences.

| Herd | # of Individuals | Median r | Factors |
|---|---|---|---|
| E | 10 | -0.02 | Low relatedness |
| C | 7 | 0.01 | Low relatedness |
| H | 10 | 0.07 | Low relatedness |
| M | 8 | 0.08 | Low relatedness |
| Q | 8 | 0.08 | Low relatedness |
| B | 10 | 0.10 | Intermediate relatedness |
| P | 9 | 0.10 | Intermediate relatedness |
| G | 11 | 0.11 | Intermediate relatedness |
| K | 7 | 0.16 | Intermediate relatedness |
| I | 5 | 0.17 | Intermediate relatedness |
| aa | 6 | 0.20 | High relatedness |
| A | 7 | 0.26 | High relatedness |
| D | 7 | 0.27 | High relatedness |
| O | 4 | 0.32 | High relatedness |
| L | 4 | 0.47 | High relatedness |

Visual inspection of the chromatograms of two highly related adult sisters (Figure 5.5) and an all-female family unit (n=4) with high genetic *r* (Figure 5.6) suggests that highly related elephants shared similar compounds in TGS, which were differentially expressed, as well as individually unique compounds.

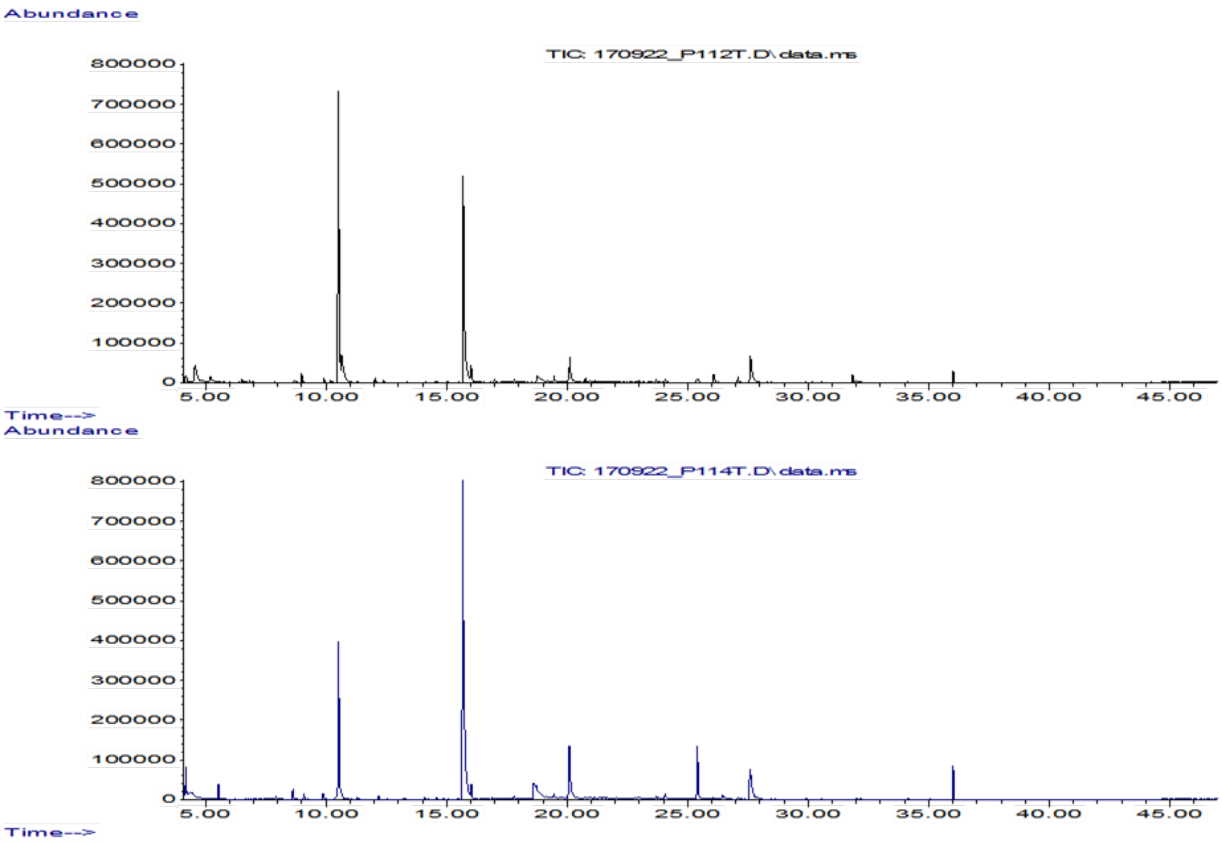


**Figure 5.5:** Total Ion Chromatogram from two closely related adult female African elephants

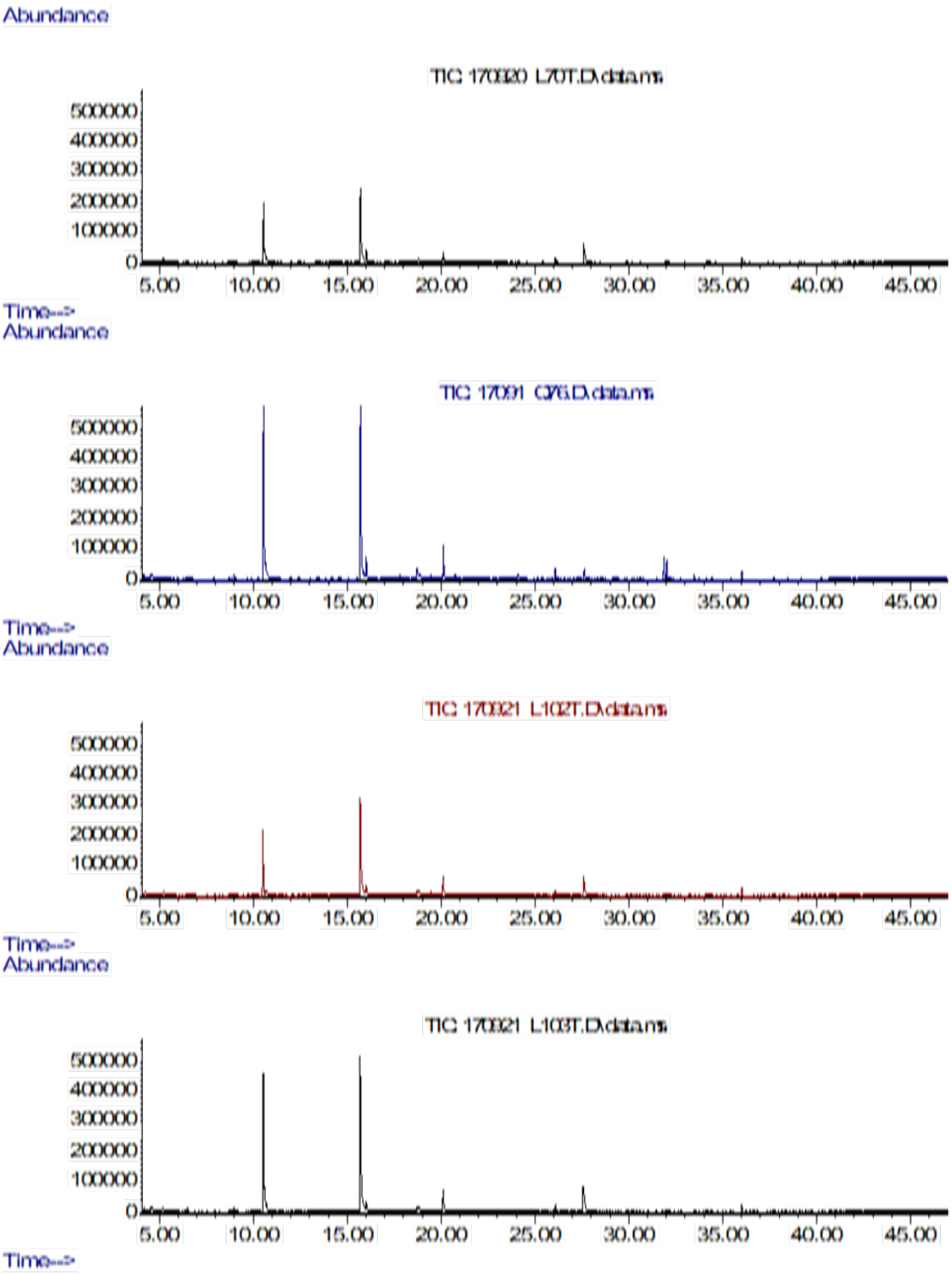


**Figure 5.6:** Total Ion Chromatogram from an all-female family unit with high genetic *r*.

**5.3.5 Genotype and overall chemical profile:** To determine whether genetic relatedness is reflected in chemical similarity, the association between Bray-Curtis similarity and pairwise genetic relatedness was tested. When all the animals were tested together, no significant

relationship was found (Mantel's r = 0.02, n=106, P = 0.18). When highly related herds were analysed, no significant relationship was found between the three categories of herds (Mantel's r = -0.024, n=30, P = 0.6). When full sister dyads at QG >0.45 were tested (Please refer to Supplementary Information Table S7.1), dyad was found to be a significant factor ($p < 0.0125$), however no significant correlation between genetic and chemical similarity for the seven dyads was found (Mantel's r = -0-048, n=14, P = 0.67).

**5.3.6 Identification of important compounds:** To identity substances that contributed towards age and herd dissimilarities across the sample population, "similarity percentages" (SIMPER, Table 5.3 and Table 5.4) was used. Compounds indicated by an asterisk (*) are of mammalian origin, derived from a biosynthetic acetate pathway (Charpentier et al. 2012). The major volatile compounds were short-chain free fatty acids, including acetic acid, pentanoic acid and hexanoic acid. Pentanoic acid, a colourless, oily liquid with a very unpleasant odour of stale cheese, is found naturally as free, or as esters in the vegetable and animal kingdoms (Goldberg & Rokem 2009). Geranyl acetone, or (E)-6,10-Dimethyl-5,9-undecadien-2-one, is a monoterpene ketone and is one of the key volatile flavour compounds of tomato, some species of mushroom and in the headspace of elephant garlic. Geranyl acetone was found to be an important chemical compound in explaining chemical differences between herds and between age categories. To the best of our knowledge this compound has not been previously reported in elephants.

**Table 5.3:** 30% of compounds (n=10) contribute towards the dissimilarity between herds.

| | RT | Putative ID | Average | Sd | Ratio | Ava | Avb | Cumsum |
|---|---|---|---|---|---|---|---|---|
| 1 | 15.71 | 1-Hexene | 0.02 | 0.03 | 0.73 | 1.08 | 0.63 | 0.05 |
| 2 | 18.73 | Acetic Acid** | 0.02 | 0.02 | 1.14 | 2.08 | 1.9 | 0.1 |
| 3 | 27.59 | Pentanoic Acid | 0.02 | 0.02 | 0.99 | 3.31 | 2.17 | 0.15 |
| 4 | 25.42 | **Benzaldehyde**, 4-methoxy-,oxime | 0.02 | 0.01 | 1.26 | 1.74 | 1.72 | 0.18 |
| 5 | 27.09 | Geranyl acetone* | 0.01 | 0.01 | 1.15 | 0.06 | 0.78 | 0.21 |
| 6 | 36.02 | Phenol, 2,4-bis(1,1-dimethylethyl) | 0.01 | 0.01 | 1.29 | 2.29 | 1.93 | 0.23 |
| 7 | 18.76 | **Acetic acid**** | 0.01 | 0.02 | 0.59 | 0.3 | 0.36 | 0.25 |
| 8 | 26.45 | Hexanedioic acid, dimethyl ester | 0.01 | 0.01 | 1.01 | 0.23 | 0.53 | 0.27 |
| 9 | 27.4 | **Hexanoic acid**** | 0.01 | 0.02 | 0.51 | 0.08 | 0.54 | 0.29 |
| 10 | 19.17 | **Hexanoic acid**** | 0.01 | 0.01 | 1.64 | 0.29 | 0.51 | 0.31 |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012), *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al, 2012.)*

**5.3.7 Chemical characterization of TGS in adult females:** This is the first time that the chemical composition of TGS in a wild population of adult female African elephants (n=40) is described. Adult females secrete TGS more frequently than adult males and younger elephants (Kioko et al. 2017). One hundred and sixty one substances were identified among all samples and seven substances were present in the controlled blanks. The corresponding peaks as well as the blanks were removed from the data. Sixty two substances were present in just one single sample and were removed and 92 substances were retained after all filtering steps.

**Table 5.4:** 30% of compounds (n=6) contribute towards the dissimilarity between five age categories.

| | RT | Putative ID | Average | SD | Ratio | Ava | Avb | Cumsum |
|---|---|---|---|---|---|---|---|---|
| 1 | 15.71 | 1-Hexene | 0.03 | 0.04 | 0.82 | 0.85 | 1.27 | 0.07 |
| 2 | 18.73 | Acetic Acid** | 0.03 | 0.02 | 1.21 | 1.36 | 1.43 | 0.12 |
| 3 | **18.76** | Acetic Acid** | 0.03 | 0.03 | 0.97 | 0.97 | 0.92 | 0.18 |
| 4 | 25.41 | **Benzaldehyde, 4-methoxy-,oxime** | 0.02 | 0.02 | 1.28 | 0.9 | 1.21 | 0.22 |
| 5 | 27.09 | Geranyl acetone* | 0.02 | 0.02 | 1.35 | 1.31 | 0.45 | 0.27 |
| 6 | 27.59 | Pentanoic acid | 0.02 | 0.03 | 0.83 | 2.96 | 2.96 | 0.31 |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012), *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

In terms of compound categories, aldehydes, ketones, carboxylic acids and aromatic compounds predominated, followed by alcohols and phenols. TGS contained more aldehydes than breath or genital secretions, and more arenes and aromatic compounds (combined aromatic compounds) than breath or genital secretions, which had similar amounts (concluding chapter). Compounds found in our African adult elephant females that have also been identified in Asian elephants, included ethanol, hexanal, furan, and cyclohexanone. Confirmed pheromones identified in African elephant adult female TGS included m-cresol, p-cresol, nerolidol as well as cyclohexanone – previously only identified in Asian elephants, and associated mostly with bulls in musth.

**Table 5.5.** Volatile organic compounds (>70% NIST matches), and the class of chemicals they belong to, detected in the temporal gland secretions (TGS) of adult female African elephants.

| | Compound | Compound category |
|---|---|---|
| 1 | 1-Butanol | Alcohol |
| 2 | 1-Octanol | Alcohol |
| 3 | 1-Pentanol | Alcohol |
| 4 | 3,7 -octadiene-2-ol | Alcohol |
| 5 | 3-Buten-2-ol, 2-methyl- | Alcohol |
| 6 | **3-Octanol** | Alcohol |
| 7 | **Ethanol** | Alcohol |
| 8 | Isoamylalcohol | Alcohol |
| 9 | Isooctanol | Alcohol |
| 10 | 1,4-Benzenedicarboxaldehyde | Aldehyde |
| 11 | 2,4 Pentadienal | Aldehyde |
| 12 | 2,4-Heptadienal, (E,E)- | Aldehyde |
| 13 | 2,6 Dimethylbenzaldehyde | Aldehyde |
| 14 | 2-Octenal | Aldehyde |
| 15 | 3-(4-tertiobutylphenyl)-propanal | Aldehyde |
| 16 | 5 Methyl furfural | Aldehyde |
| 17 | **Benzaldehyde** | Aldehyde |
| 18 | **Hexanal*** | Aldehyde |
| 19 | N-Hexanal | Aldehyde |
| 20 | Cyclopropane | Alkane |
| 21 | **Decane*** | Alkane |
| 22 | Dodecane* | Alkane |
| 23 | Nonane | Alkane |
| 24 | Octadecane | Alkane |
| 25 | Tetradecane* | Alkane |
| 26 | 1,3 Pentadiene | Alkene |
| 27 | 1-Hexene | Alkene |
| 28 | 1-Pentene | Alkene |
| 29 | 2-Methyl-4-(4-methoxyphenyl)-2-pentene | Alkene |
| 30 | 2-Pentene, (E)- | Alkene |
| 31 | Benzylamine | Amine |
| 32 | 1,4-Di-n-butylbenzene | Arene |
| 33 | Benzene | Arene |
| 34 | Butylbenzene | Arene |
| 35 | (+,-)-N-NORCULARICINE | Aromatic compound |
| 36 | Delta-cadinene | Aromatic compound |
| 37 | 2-(Dimethylamino)-3-phenylbenzo[b]thiophen | Aromatic compound |
| 38 | 2-Methyl-5,12-dithianaphtho[2,3-b]quinoxaline | Aromatic compound |
| 39 | 3,3,6,9,9,10-Hexamethyl-2,10-diazabicyclo[4.4.0]-1-decene | Aromatic compound |
| 40 | M-Cymene, 5-tert-butyl- | Aromatic compound |
| 41 | Acetic acid* ** | Carboxylic acid |
| 42 | **Benzoic acid*** ** | Carboxylic acid |
| 43 | Butanoic acid* | Carboxylic acid |
| 44 | **Caproic acid**/hexanoic acid**** | Carboxylic acid |
| 45 | Hexadecanoic acid** | Carboxylic acid |
| 46 | Hexanedioic acid | Carboxylic acid |
| 47 | **Hexanoic acid*** ** | Carboxylic acid |

| | Compound | Compound category |
|---|---|---|
| 48 | Pentanoic acid | Carboxylic acid |
| 49 | **Furan** | Cyclic ether |
| 50 | Oxirane | Cyclic ether |
| 51 | .Gamma. Hexalactone | Ketone |
| 52 | 2(3H)-Furanone | Ketone |
| 53 | 2-Cyclohexen-1-one | Ketone |
| 54 | 3,7-octadiene-2-one | Ketone |
| 55 | 3-methylene-6-hepten-2-one | Ketone |
| 56 | 6-Methyl-3,5-Heptadien-2-One | Ketone |
| 57 | **Cyclohexanone *** | Ketone |
| 58 | Ethanone | Ketone |
| 59 | Geranylacetone* | Ketone |
| 60 | 5,9-Undecadien-2-one, 6,10-dimethyl-, (E)- | Monoterpene ketone |
| 61 | 2-Pyrrolidinone | Nitro-ketone |
| 62 | 2-Allyl-4-(tert-butyl)phenol | Phenol |
| 63 | Chavicol* | Phenol |
| 64 | I-H-Inden-5-ol | Phenol |
| 65 | **M cresol*** | Phenol |
| 66 | **P cresol*** | Phenol |
| 67 | **Nerolidol** | Sesquiterpenoids |
| 68 | Linalool* | Terpenoid |

***Bold compounds*** *are confirmed semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al 2002, 2005, 2006, 2012, 2016), *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

## 5.4 Discussion:

This study was motivated by the fact that no information exists regarding TGS function and chemistry in African elephant females, which is perplexing given that they frequently secrete TGS, notably during social interactions. To date, much of the chemosensory research in mammals has focused on male sexual signalling to attract females and /or to mediate competition between rival males (Clutton-Brock & McAuliffe 2009) as promulgated by Darwin's theory of sexual selection (Darwin 1871). This has also been the case with chemical research in elephants, which has primarily focused on captive Asian elephants and sexual signalling in captive Asian bulls in musth (Goodwin et al. 2012, Schulte & Rasmussen 1999, Rasmussen 1998, 2000, Rasmussen & Perrin 1999, Rasmussen & Greenwood 2003, Rasmussen, Hess & Haight 1990), probably due to male-biased sexual dimorphism and scent-signalling behaviour in Asian elephants. However, female mammals also use scent signalling for sexual attraction, mediation of female competition and cooperation, and to facilitate maternal behaviour (Glutton Brock & Huchard 2013). Chemical signalling and how it governs behaviour and sociality in

female African elephants is interesting as females are philopatric, live in matrilineal, multi-tiered, fission-fusion groups marked by linear dominance hierarchies, reciprocal cooperation, coalitions, and differentiated social relationships between group members which are indicative of social complexity (Lukas & Clutton-Brock 2018).

In this study, TGS encoded for age in African elephants. Age class differences in TGS chemical composition are likely due to hormonal variations and varied physical development, and where diet and gut bacteria are known to contribute to odour profiles (Kean & Chadwick 2011). Studies suggest that frequency of African elephant TGS secretion varies with gender and age (Kioko et al. 2017), while age has been correlated with chemical profile in other mammals including rhinos (*Ceratotherium simum*) (Marneweck et al. 2017), owl monkeys (*Aotus nancymaae*) (MacDonald et al. 2008), pandas (*Ailuropoda melanoleuca*) (Zhang et al. 2008), mandrills (*Mandrillus sphinx*) (Setchell et al. 210) and otters (Kean et al. 2011). Moreover, the SIMPER analyses identified five compounds that explain 30% of the cumulative differences among age categories – of which three compounds (acetic and pentanoic acid, and geranyl acetone) are compounds that are confirmed pheromones (www.pherobase.com) in African mammals such as lion (*Panthera leo*), leopard (*Panthera pardus*), Africa wild dog (*Lycaon pictus*) and cheetah (*Acinonyx jubatus*). It is probable that TGS also carries a signal for sex as our results tended towards significance. The absence of adult males in this sample population may have impacted the sensitivity of the analysis to differences between male and female individuals. Sex was clearly a significant factor separating adult females and males in elephant urine (see Chapter 4) and it is likely that this would be reflected in TGS, given a sample with equivalent number of mature bulls and olfactory signal convergence in elephants (Rasmussen 1998).

Unexpectedly, TGS did not appear to encode for genetic relatedness in African elephants, not at the population, herd, or individual level. Instead, TGS encoded individual identity in the sample population, with chemical profiles varying in compound diversity and abundance across sexes, ages and relatedness levels. Olfactory signals for individual identity have been recorded in hyenas (Burgener et al. 2009) and raccoons (Kent & Tang-Martinez 2014), and the ability to discriminate individual odours has been recorded in a diversity of species, including ring-tailed

lemurs, golden hamsters (*Mesocricetus auratus*), raccoons, dwarf mongoose (*Helogale parvula*), meerkat (*Suricata suricatta*), white rhino (*Ceratotherium simum*), penguins (*Spheniscus humboldti*), and kittiwakes (*Rissa tridactyla*) (Marneweck et al. 2017, Palagai & Dapporto 2006, Johnston et al. 1993, Kent & Tang-Martinez 2014, Coffin et al. 2011, LeClaire et al. 2011). Recognition requires distinct olfactory cues and TGS appeared individually distinctive, when visually comparing the number and intensity of compounds across a subset of the sample population. Highly related individuals (sisters and herds) shared a subset of chemicals which were expressed differently, and individuals had compounds in their TGS unique to their individual odour profile. Individual odour signatures may result from environmental and /or genetic factors, and may be expressed by the presence and absence of compounds as well as by the amount expressed of each compound (Sun & Muller Schwarze 1998, Apps 2013).

This study expected that herds would share a group odour as previous research on elephants in Amboseli National Park showed that genetic patterns among elephant herds seemed important to inclusive fitness benefits underlying elephant sociality, and hierarchical structuring was correlated to kinship (Archie et al. 2006). Furthermore, in fission-fusion societies, where females split into multiple subgroups, herd-specific odour may facilitate long-term group stability, and has been confirmed in another fission-fusion mammal, namely the spotted hyena (Burgener 2008). Genetic relatedness has also been correlated with chemical similarity (known as Odour-Gene Covariance) in other species from primates to rodents (Boulet et al. 2009, Tzur et al. 2009). Given that herd- and individual odour had no correlation with genetic relatedness in this studies sample population across relatedness levels, allows for the possibility that bacteria may account for the main differences in group odour. Bacteria are a significant factor in mammalian chemical communication, and bacterial activity has been linked to VOCs and odour profiles in anal glands in meerkats, in the inguinal pouch of rabbits *Oryctolagus cuniculus* (Merritt et al. 1982), in anal sacs of the red fox (*Vulpes vulpes*), in exocrine secretions of beavers (Muller-Schwarze & Heckmann 1980) and in scent marks in spotted hyena (Albone et al. 1974, Theis et al. 2013). The fermentation hypothesis for chemical communication (Albone et al. 1974, Gorman 1976) suggests that scent glands harbor symbiotic bacteria that decompose organic material and produce VOCs, which may contribute to intra-

individual recognition in mammals (Archie & Theis 2011). Bacteria, found in host urine, faeces or in products from specialized sebaceous or apocrine scent glands, are suggested to encode information about sex, age, breeding condition, health, diet, dominance rank and social relationships of their hosts.

A number of important compounds explaining differences between herds were also identified: these were acetic acid, hexanoic acid, pentanoic acid, benzaldehyde, geranyl acetone and phenol. Notably, semiochemicals found in the buccal secretions of the dwarf hamster, presumed to be a signal for identity, sex, and breeding condition (Singer et al. 1980), included these compounds, notably hexanoic acid, acetic acid, butanoic acid and pentanoic acid. These compounds have further been confirmed to have a pheromone function in the chemical communication among diverse African mammals, including lion, red hartebeest (*Alcelaphus buselaphus caama)*, wild dog, springbok (*Antidorcas marsupialis*), giraffe (*Giraffa Camelopardalis*), cheetah (*Acinonyx jubatus*), and black backed jackal *Canis mesomelas* (please refer to Pherobase.com for a complete overview).

In order to assist with compound selection for future verification and bioassays, chemical compounds of interest were noted that either confirm or challenge previous findings in elephants. Compounds identified in adult female TGS have been associated with communication of individual-specific information in a diversity of other mammals. For example, phenols such as m-cresol and chavicol has been identified in castoreum exudate in beavers, which utilize caustoreum for territory marking (Burger 2005). Both the African bontebok (*Damaliscus dorcas dorcas*) and black-tailed deer (*Odocoileus hemionus. columbianus)* use interdigital and forehead secretions respectively for marking territory (Burger 2005). African elephant females shared a number of compounds with antelope, such as linalool, dodecane, decane, benzaldehyde, hexanal and 6-methyl-3,5-heptadien-2one with white tailed deer. Elephants also shared VOCs with territorial carnivores with a number of TGS semiochemicals also reported in the scent-marking fluid of African lion: cyclohexanone, linalool, 1-Octanol, acetic acid, and benzaldehyde (Soso & Koziel 2017).

With regards to the published literature on elephant semiochemicals, previously published compounds (Goodwin et al. 1999) were also confirmed in this study, and included p-cresol,

phenol, and benzoic acid. TGS contained sesquiterpenes (nerolidol) in captive African female elephants in the USA (Goodwin et al. 2002), and were also found in the current study. None of the published Asian elephant pheromones (Rasmussen et al. 1996, 2001, Rasmussen & Krishnamurthy 2000) were identified in our samples, possibly due to the absence of mature males and which have tended to be the focus of captive *Elephas* research. Nevertheless, compounds previously identified and confirmed in Asian elephants (Rasmussen 2001, 1998) were also identified here and included phenol, pentene, ethanol, hexanal, cyclohexanone, acetic acid, and furan.

A surprising finding was the presence of cyclohexanone in adult female TGS, a compound thought to previously be associated with Asian male elephants in musth. However, in other species cyclohexanone is associated with attraction, locomotion, stimulation and inhibition (Soso & Koziel 2017). Compounds associated with African male elephants (Rassmussen et al. 1996) were also identified in the sampled females, and included benzoic acid, hexanoic acid and hexadecanoic acid. In this study, compounds shared in female urine and TGS in African elephants (see Chapter 4) included phenols, octanol, benzoic acid, butanoic acid, cyclohexanone, benzaldehyde, furans, decanes and benzene derivatives.

## 5.5 Conclusion

These preliminary results suggest that TGS volatiles carry variable (age) as well as fixed (individual identity, possibly sex) information in African elephants as has been found in other mammals. Further, results suggest the possibility of a group odour among African elephants that is not determined by genetic relatedness, and which may be the result of bacteria. It is currently not known the degree to which bacteria contribute to the production of individual or group odour in African elephants and analysis of the various elephant microbiomes will contribute towards a better understanding of this. Specifically if bacteria are found to play a key role in elephant odour, the use of antibiotics to suppress odourant production relating to sexual signalling from scent glands could be of interest in captive settings. Future research should explore olfactory signals for individual identity further, by sampling repeat replicates per individual across seasons. Also, the extent to which TGS contains signals reflecting

reproductive states and dominance hierarchies needs to be examined further in a semi-natural setting where dominance rank is known and physiological condition of adult females can be tracked. For the first time, the chemical composition and diversity of wild African elephant adult females TGS is putatively described. Specifically, if the presence of cyclohexanone (a compound associated with Asian bull elephants) is verified, the signalling role of TGS may need to be revaluated in the scientific literature for both Asian and African elephants, with regards to male-male, male-female, and possibly female-female reproductive signalling. Further, to understand the significance of results, behavioural bioassays are required to examine the functional role of important compounds identified here to the behaviour of adult females.

## 5.6 Limitations:

Identifying the chemical information contained in secretions of the temporal gland is essential for understanding communication in African elephants, and may have important implications for managing African elephants both in captive and wild settings (Schulte et al 2007). The results presented here should be interpreted with caution as they reflect a once-off sampling event from a translocation targeting family groups. Potential confounding factors may include differences in gut flora and behavioural complexity due to the stress of the translocation. It is hoped that the preliminary results presented here will provide a framework for future research on African elephant adult female semiochemicals, chemical communication and behaviour and contribute to this endangered mammal's conservation and management both in natural and in sanctuary settings.

## 5.7 References

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# Chapter 6: Characterizing the chemical composition of African elephant buccal and genital secretions using Solid Phase Microextraction (SPME) and Multidimensional Gas Chromatography-Mass Spectrometry (GC_MS)

*"For the first half of geological time our ancestors were bacteria. Most creatures still are bacteria, and each one of our trillions of cells is a colony of bacteria."*

*Richard Dawkins*

**Abstract:**

The social lifestyle of elephants suggests that beside acoustic signals, they use scent to communicate information, for conspecific recognition, mate choice and dominance rank establishment. African and Asian elephants of both sexes engage in distinctive trunk-tip behaviours when inspecting conspecific genitalia, temporal glands, mouth, ears and feet. This suggests that both buccal and genital secretions may play an important olfactory role in intraspecific reproductive and social signalling. Surprisingly little has been published on the chemical composition and function of genital and buccal secretions in female African elephants. Using gas chromatography–mass spectrometry (GC_MS), the chemical composition of buccal and genital secretions from 113 individually identified African elephants from 15 herds sampled during a translocation in Malawi were investigated. All individuals were genotyped using eighteen microsatellite markers and pairwise genetic distance comparisons conducted to estimate genetic similarity between each pairwise dyad. GC_MS, genetic analyses and an array of statistical methods were used to determine whether genital and buccal odour encode sex, age, relatedness and identity in African elephants. No correlation was found between genetic relatedness and chemical profile of genital and buccal secretions at the population or herd level. Instead, both buccal and genital secretions encoded individual identity. Buccal secretions also encoded age, while genital secretions tended towards significance for sex. "Herd" was found to be a significant factor in explaining chemical differences between groups for both genital and buccal odours, and a number of short chain fatty acids known to result from bacterial fermentation identified. This suggests that bacteria may play a role in group odour in African elephants. For the first time, labial and buccal secretions in 40 adult females are characterised. Compounds of mammalian origin were identified in buccal secretions, which contained mainly esters, as well as alcohols, arenes and alkanes. Labial secretions comprised predominantly alcohols, esters, and ketones. The SIMPER analysis extracted fatty acids, wax esters and phenols as compounds of significance in explaining group differences in odour, which further points to a possible bacterial role in African elephant odourants. Putative identifications of volatile fatty acids presented here may prove useful in the search of an oestrus/preovulatory pheromone in labial secretions in African elephants, while the vast cocktail of volatile and semi-volatile compounds identified in elephant buccal secretions may provide a first step to test for olfactory signals for pregnancy or ovulation in elephant females, which is useful for management of the species in sanctuary environments.

**Key words**: African elephant, bacteria, buccal secretions, GC_MS, genital secretions, identity, olfaction, relatedness, semiochemicals

## 6.1 Introduction

African and Asian elephants of both sexes engage in distinctive trunk-tip behaviours when inspecting conspecific genitalia, temporal glands, mouth, ears and feet. Extensive demographic and behavioural records from Kenya show that African elephants engage in frequent affiliative, tactile behaviour, with social rubbing during greetings, test-mouthing between non-relatives especially males, and social learning with calves touching mouths of older individuals (Poole & Granli 2011). Elephants consequently appear to use different odours in different social and sexual contexts, with specific behaviours focused on specific glands (such as urine, dung, and temporal gland investigated for reproductive status, trunk-to-mouth reaches during male greetings, or trunk-to-mouth reassurance between a calf and a female conspecific), while a combination of behaviours, sounds and scents are expressed during ritualized greeting ceremonies. This suggests that both buccal and genital secretions play an important olfactory role in intraspecific reproductive and social signalling. Research has mainly focused on urinary and temporal gland signatures for musth and oestrus in elephants, and in urine and specialized scent glands in mammals in general.

Extensive research on Asian elephant semiochemistry suggests that bulls emit chemical signals in breath (Rasmussen & Krishnamurthy 2000), while in the African elephant, females approaching ovulation receive significantly more urogenital checks by other females (Meyer et al. 2007) than luteal phase females. The extent to which African elephants transmit information via genital and buccal secretions is less well understood. No publications exist that describe buccal and genital semiochemistry in wild African elephants. Elephant buccal and genital secretions are likely to signal social information regarding fixed (gender, identity) and variable factors (age, rank, health, reproductive state) during short-term behavioural interactions such as during greeting ceremonies between herds, and encounters between males.

African elephants breed throughout the year. Females live in multi-female fission-fusion groups characterized by female philopatry, male dispersal and intrasexual dominance hierarchies (Wittemyer et al. 2005). Elephants emit chemical signals through urine, dung, labial and penile mucus, trunk and olfactory regions, exhalant breath, ears, temporal gland and toes

(Schulte et al. 2007, Rasmussen et al. 1997, Lamps et al. 2001). Research on elephant semiochemistry has tended to focus on Asian elephant sexual signalling in urine, TGS and breath (Rasmussen et al. 1988, 1986, 2001, Rasmussen & Schulte 1998, Goodwin et al. 1999), and has revealed a similarity of compounds between musth bull TGS, urine and breath, with major compound classes including hydrocarbons, ketones, dienes, furans and aldehydes (Rasmussen 1998). Based on behavioural studies in Asian elephants (*Elephas maximas*), the urinary pheromone Z7-12:Ac signals impending oestrus in elephant females during the preovulatory phase, and Asian elephant females inspect the urogenital region of conspecific females more frequently in the oestrus than in the luteal phase of their cycle (Rasmussen & Krishnamurthy 2000). In response to the oestrus signal, male Asian elephants flehmen, transferring the compound from the trunk-tip to the vomeronasal organ in the roof of the mouth. Females show lower rates of flehmen and it is suggested that flehmen in females occurs in a social context (Rasmussen 1998). The urogenital area in elephants is an important source of female-to-female and female to male sexual signalling (Rasmussen & Schulte 1998, Bagley et al. 2006, Meyer et al. 2008). Behavioural studies suggest that female African elephant labial secretions may encode an oestrus signal (Meyer et al. 2008), however the chemical identity of the pheromone is yet to be confirmed. Genitals, in addition to temporal gland, urine and faeces, are monitored intra-and intersexually and it can be expected that at least some of the chemical compounds among the different glands will be shared.

Characterising buccal and genital semiochemistry in African elephants will improve our understanding of elephant social and sexual behaviour and communication. In other mammals for example, research on rhesus monkeys (*Macaca mulatta*) suggested that volatile fatty acids in vaginal fluids function as pheromones (Michael et al. 1971), and that these aliphatic acids (such as acetic, propionic, isobutyric, butyric, isovaleric, and isocaproic acids) arise as byproducts of the fermentation of vaginal flora. These were termed “copulins” by some authors, and while their existence is still debated (Apps et al. 2015), studies appear to suggest that they increase the frequency of mounting behaviour and ejaculation in certain males. Notably these are suggested to also occur in human vaginal secretions, fluctuating across the menstrual cycle, suppressed by contraceptives and waning with menopause (Drea 2014).

In humans, breath may provide cues to pregnancy or to help new-borns recognize their mothers (Bikov et al. 2011). Semiochemicals found in the buccal secretions of the dwarf hamster (*Phodopus sungorus*), presumed to be a signal for identity, sex and breeding condition (Singer et al. 1980), were also identified in female TGS (Chapter 5), and included volatile aliphatic acids such as hexanoic, acetic, butanoic and pentanoic acids. Buccal secretions in both dwarf and golden hamsters (*Mesocricetus auratus*) appear to encode for sex, identity, and female breeding condition, and may also influence the development of pups (Burger 2005).

The present study forms part of a greater body of research investigating olfaction and chemical communication in a socially complex and highly cognitive mammal, the African elephant, *Loxodonta africana*. In this study, SPME-GC_MS, genetic analyses and an array of statistical methods were used to explore whether chemical compounds found in genital and buccal secretions encode, sex, age, relatedness and identity in African elephants. Genital and buccal secretions were then characterized for 40 adult females. The study explores whether a) genital secretions encode for sex and age b) buccal secretions encode sex, age and group membership and c) genetic relatedness is correlated with chemical similarity in both.

**6. 2 Methods**: Please refer to Chapter V for the methodology, which is the identical unless otherwise indicated.

**6.2.1 Genetic analyses for mother-offspring pairs:** Cows and calves were genotyped for maternity analysis using the program Cervus 2.0 (Marshall et al. 1998) to verify if the mother-offspring dyad was genuine. Cervus uses a likelihood-based approach to assign parentage combined with simulation of parentage analysis to determine the confidence of parentage assignments. Cervus was also used to calculate observed and estimated heterozygosity.

## 6.3 Results:

**6.3.1 Genetic and chemical data:** DNA from 113 elephants was sampled. For the age/sex population structure, please refer to the methods in Chapter 5. Using Cervus LOD and Delta scores/Match probability scores, 13 mother-offspring dyads were identified for further targeted analysis. Females were classified as adult if they were reproductively active. For chemical and

relatedness correlations, pairwise estimators of QG >0.4 were used and yielded 14 adult sister dyads (S6.1). From the maternity analysis in CERVUS, 72 of 76 mother-calf dyads were retained. Four dyads displayed negative LOD scores (Supplementary Information Table S5.2).

**6.3.2 GC AlignR Alignment results:** Genital samples were collected for analysis from 109 elephants. After removing compounds from the blank and those present only in one sample, 308 genital substances were retained after all filtering steps. Buccal samples were collected from 106 elephants. After removing compounds from the blank and those present only in one sample, 370 buccal substances were retained after all filtering steps.

**6.3.3 Group differences.** Multivariate statistical analyses of the relative proportion of each substance sampled from the 15 herds suggested significant differences in chemical profiles of genital and buccal secretions among herds, as well as among age categories in buccal secretions (Table 6. 1). Sex tended towards significance in genital secretions.

**6.3.4 Genotype and chemical profile:** To determine whether genetic relatedness was reflected in chemical similarity, the association between Bray-Curtis similarity and pairwise genetic relatedness was tested among a priori defined groups including related herds, adult sisters >0.45 QG, and mother-offspring dyads. No genetic correlation was found for buccal nor genital secretions and genetic relatedness at the population or herd level (Table 6.1). A significant results was recorded for labial secretions among adult sisters (n=14), this was however not highly significant and requires further evaluation given the small sample size.

**Table 6.1:** ANOSIM and Spearman Rank results for sample population for genital and buccal secretions in African elephants.

| | **Genital (n=109)** | **Buccal (n= 106)** |
|---|---|---|
| ANOSIM | | |
| **Herd** | ANOSIM, global $r^2$ =0.2, p<0.0001*** | ANOSIM, global $r^2$ =0.15, p < 0.0001*** |
| **Age** | ANOSIM, global $r^2$ = 0.05 p< 0.16 | ANOSIM, global $r^2$ = 0.06, p< 0.009** |
| **Sex** | ANOSIM, global $r^2$ = 0.02, p<0.07 | ANOSIM, global $r^2$ = 1.21, p<0.21 |
| Spearman Rank Correlation | | |
| **Population** | Mantel's r = 0.006, n=109, P =0.4 | Mantel's r = 0.53, n=106, P = - 0.02 |
| **Herd** | Mantel's r = - 0.02, n=30, P=0.61 | Mantel's r = -0.024, n=30, P = 0.63 |
| **Adult sister dyads** | Mantel's r = 0.19, n=14, P=0.04* | Mantel's r = 0.10, n=14, P = 0.16 |
| **Mother-offspring dyads** | Mantel's r = -0.14, n=18, P= 0.97 | Mantel's r = 0.012, n=18, P = 0.38 |

Significant results (p<0.01) are indicated with an *****.

**6.3.5 Important substances:** To identify substances that contribute towards age and herd dissimilarities across the sample population, “similarity percentages” (SIMPER, Table 6.2 and Table 6.3) was used. The major compounds for genital differences among herds included four phenols, alcohol and two esters, as well as three previously identified pheromones in African elephants: m-cresol, p-cresol and acetic acid (Table 6.2). In buccal secretions, the SIMPER analysis also identified short chain fatty acids (SCFAs) such as acetic acid, tetradecanoic acid, dodecanoic acid, butanoic acid as well as undecanoic acid and pentanoic acid for chemical differences among herds. For age differences in buccal secretions, SIMPER identified acetic acid, pentanoic acid, Phenol2,4 and 1-Hexanol, as well as Oxime, 1-H_Indan-5-ol, and Octadecanoic acid as shared compounds.

**Table 6.2:** 30% of genital compounds (n=8) contribute towards differences among herds (SIMPER)

| | RT | Putative ID | average | sd | ratio | ava | avb | cumsum |
|---|---|---|---|---|---|---|---|---|
| **1** | 27.54 | Pentanoic acid | 0.02 | 0.02 | 1.29 | 0.91 | 1.44 | 0.03 |
| **2** | 31.87 | **Phenol, 4-methyl**- ** | 0.02 | 0.02 | 1.08 | 1.96 | 1.18 | 0.06 |
| **3** | 18.73 | **Acetic acid* ** ** | 0.02 | 0.02 | 1.25 | 1.5 | 0.43 | 0.08 |
| **4** | 32.02 | **Phenol, 3-methyl- ** ** | 0.02 | 0.02 | 1.12 | 1.64 | 0.98 | 0.11 |
| **5** | 18.64 | 1-Hexanol, 2-ethyl**- ** ** | 0.02 | 0.02 | 1.08 | 0.31 | 1.31 | 0.16 |
| **6** | 25.45 | **Hexanoic acid** | 0.01 | 0.01 | 1.4 | 0.64 | 1.41 | 0.2 |
| **7** | 23.78 | Pentanedioic acid, dimethyl ester | 0.01 | 0.01 | 0.99 | 0.23 | 0.75 | 0.28 |
| **8** | 36.02 | Phenol, 2,4-bis(1,1-dimethylethyl)- | 0.01 | 0.01 | 1.36 | 1.36 | 1.04 | 0.31 |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012, 2016) *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

**Table 6.3:** 30% of buccal compounds (n=16) contribute towards chemical differences among a) herds (n=16) and b) age (n=17) (SIMPER)

a) Herd (n=16)

| | RT | Putative ID | average | sd | ratio | ava | avb | cumsum |
|---|---|---|---|---|---|---|---|---|
| **1** | 27.47 | Pentanoic acid | 0.02 | 0.01 | 1.16 | 1.26 | 1.88 | 0.03 |
| **2** | 25.81 | Undecanoic acid, 10-methyl-, methyl ester** | 0.01 | 0.01 | 1.38 | 1.29 | 0.90 | 0.07 |
| **3** | 18.61 | **Acetic acid**** | 0.01 | 0.01 | 0.96 | 0.95 | 0.44 | 0.09 |
| **4** | 18.75 | Decyl Aldehyde | 0.01 | 0.01 | 0.88 | 0.59 | 0.54 | 0.13 |
| **5** | 18.70 | 1-Hexanol, 2-ethyl- ** | 0.01 | 0.01 | 1.38 | 1.31 | 1.48 | 0.14 |
| **6** | 30.16 | **Tetradecanoic acid**, methyl ester** | 0.01 | 0.01 | 1.41 | 1.10 | 1.16 | 0.17 |
| **7** | 25.75 | **Dodecanoic acid**, methyl ester** | 0.01 | 0.01 | 0.72 | 0.37 | 0.55 | 0.18 |
| **8** | 15.60 | Nonanoic acid, 9-oxo-, methyl ester | 0.01 | 0.01 | 0.61 | 0.23 | 0.55 | 0.20 |
| **9** | 34.07 | Hexadecanoic acid, methyl ester** | 0.01 | 0.01 | 1.35 | 2.03 | 1.93 | 0.21 |
| **10** | 27.54 | Butanoic acid, 3-methyl- | 0.01 | 0.01 | 0.58 | 0.45 | 0.33 | 0.22 |
| **11** | 34.54 | 7-Hexadecenoic acid, methyl ester, (Z)- | 0.01 | 0.01 | 1.33 | 0.77 | 0.80 | 0.24 |
| **12** | 36.04 | Phenol, 2,4-bis(1,1-dimethylethyl)- | 0.01 | 0.00 | 1.97 | 1.66 | 1.01 | 0.25 |
| **13** | 18.55 | Nonanoic acid, methyl ester** | 0.01 | 0.01 | 0.72 | 0.25 | 0.54 | 0.26 |
| **14** | 21.02 | **Decanoic acid**, methyl ester ** | 0.01 | 0.01 | 1.04 | 0.55 | 0.33 | 0.27 |
| **15** | 18.80 | **Acetic acid*** ** | 0.01 | 0.01 | 0.75 | 0.30 | 0.39 | 0.28 |
| **16** | 23.73 | 2(3H)-Furanone, 5-ethyldihydro- | 0.01 | 0.00 | 1.54 | 0.62 | 0.45 | 0.29 |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012, 2016, *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

b) Age categories (n=17)

| | RT | Putative ID | average | sd | ratio | ava | avb | cumsum |
|---|---|---|---|---|---|---|---|---|
| **1** | 27.50 | Pentanoic acid | 0.01 | 0.02 | 0.90 | 0.91 | 0.71 | 0.04 |
| **2** | 34.07 | Hexadecanoic acid, methyl ester** | 0.01 | 0.01 | 1.39 | 1.44 | 1.40 | 0.06 |
| **3** | 18.70 | 1-Hexanol, 2-ethyl- E ** | 0.01 | 0.01 | 1.22 | 1.32 | 0.94 | 0.08 |
| **4** | 27.54 | Butanoic acid, 3-methyl- | 0.01 | 0.02 | 0.87 | 0.70 | 0.76 | 0.10 |
| **5** | 25.81 | Undecanoic acid, 10-methyl-, methyl est ** | 0.01 | 0.01 | 1.12 | 0.67 | 0.83 | 0.12 |
| **6** | 30.16 | **Tetradecanoic acid**, methyl ester** | 0.01 | 0.01 | 1.23 | 0.71 | 0.80 | 0.13 |
| **7** | 34.11 | Hexadecanoic acid, methyl ester** | 0.01 | 0.01 | 0.73 | 0.63 | 0.42 | 0.15 |
| **8** | 18.80 | **Acetic acid*** | 0.01 | 0.01 | 0.77 | 0.45 | 0.57 | 0.19 |
| **9** | 18.75 | Decyl aldehyde | 0.01 | 0.01 | 0.72 | 0.25 | 0.68 | 0.20 |
| **10** | 26.00 | 1H-Inden-5-ol, 2,3-dihydro- | 0.01 | 0.01 | 1.33 | 0.83 | 0.92 | 0.21 |
| **11** | 34.54 | 7-Hexadecenoic acid, methyl ester, (Z)- | 0.01 | 0.01 | 0.98 | 0.48 | 0.55 | 0.23 |
| **12** | 15.60 | Nonanoic acid, 9-oxo-, methyl ester * | 0.01 | 0.01 | 0.81 | 0.50 | 0.41 | 0.24 |
| **13** | 36.04 | Phenol, 2,4-bis(1,1-dimethylethyl)- | 0.01 | 0.01 | 1.35 | 0.83 | 1.06 | 0.25 |
| **14** | 25.51 | Oxime-, methoxy-phenyl-_ | 0.01 | 0.01 | 1.15 | 0.40 | 0.75 | 0.26 |
| **15** | 37.99 | 9-Octadecenoic acid (Z)-, methyl ester** | 0.01 | 0.01 | 1.27 | 0.75 | 0.60 | 0.27 |
| **16** | 18.40 | Nonanoic acid*, methyl ester | 0.01 | 0.01 | 0.58 | 0.43 | 0.30 | 0.28 |
| **17** | 18.90 | Amyl Hexanoate | 0.01 | 0.01 | 0.67 | 0.41 | 0.24 | 0.31 |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012, 2016, *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

**6.3.6 Chromatograms:** Visual inspection of sample chromatograms (sister dyad) and highly related herd (Herd L) suggested that both buccal and genital secretions differed between highly related adult sisters, and between members of a highly related all female family unit (mother and offspring aged 40, ten, six and one years) both qualitatively and quantitatively (Figures 6.1 & 6.2).

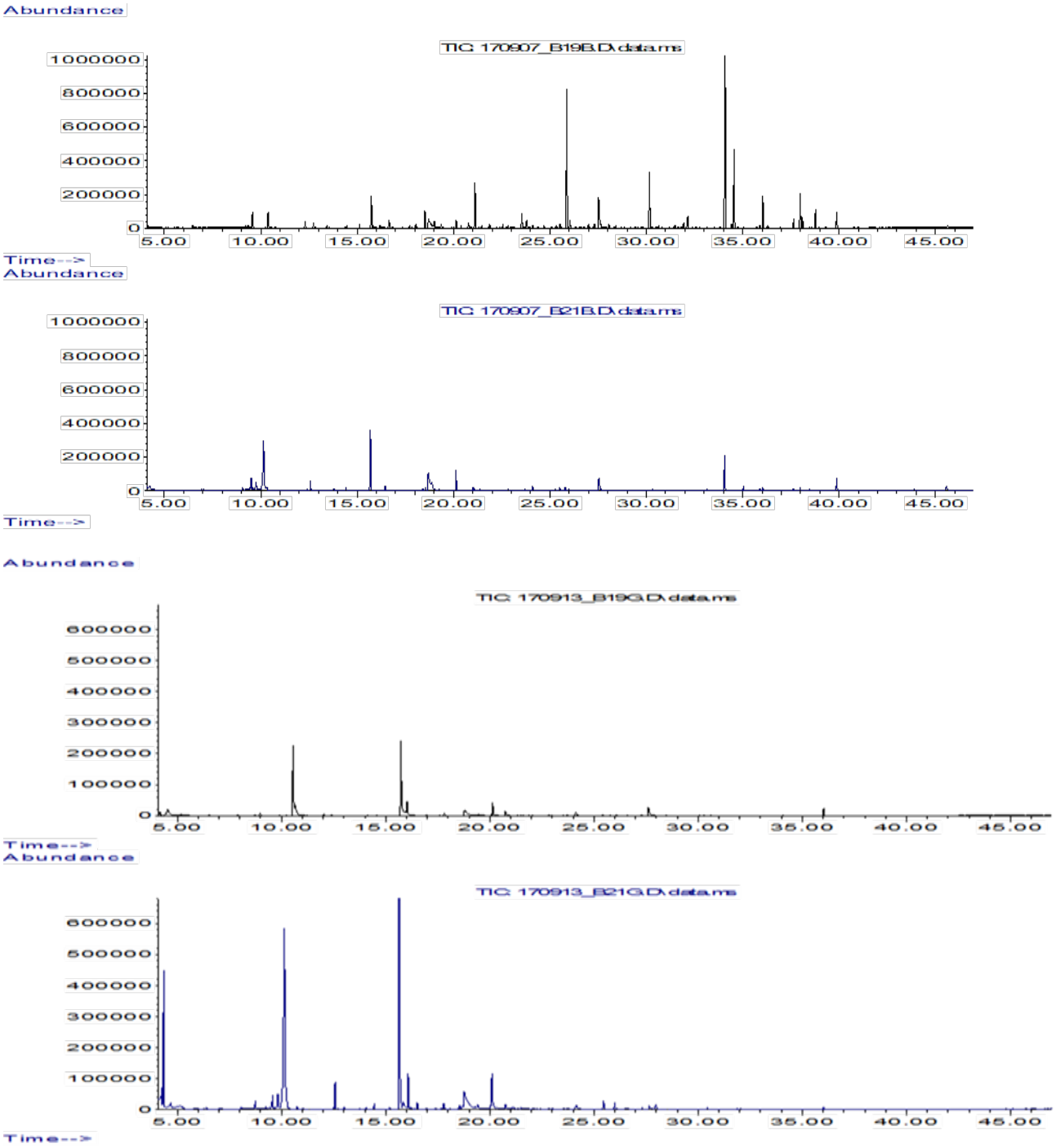


**Figure 6.1**: Representative chromatograms of buccal (top) and genital (bottom) secretions produced by two highly related adult African elephant sisters

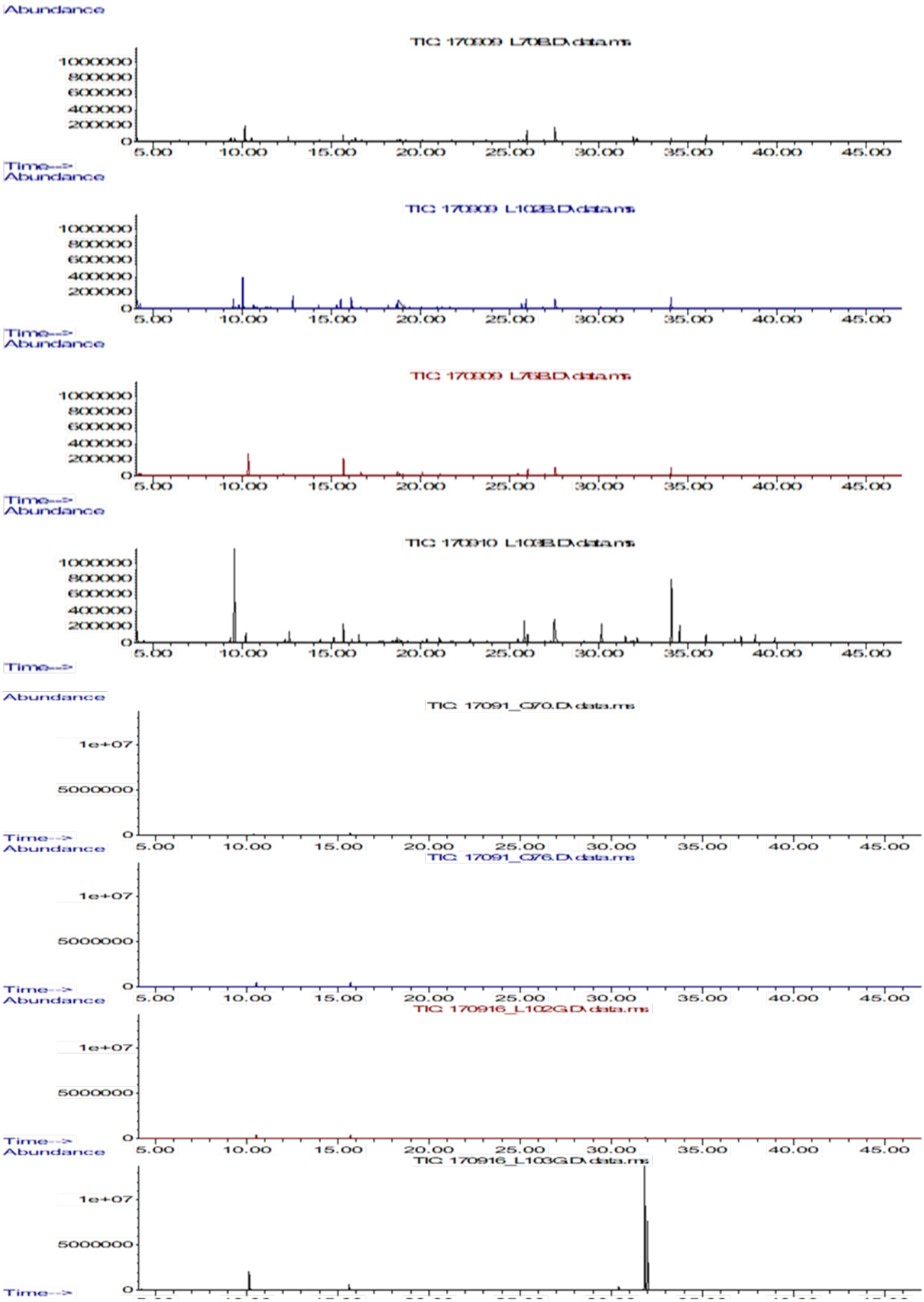


**Figure 6.2:** Representative chromatograms of buccal (top) and genital (bottom) secretions of a family herd comprising adult, subadult, calf and infant African elephant females

**6..3.7 Putative chemical characterization of labial secretions in adult females**: Labial secretions of adult elephant females contained alcohol, aromatic compounds, arenes, ketones, alkanes, and phenols were identified as the major chemical classes (Table 6.4). Confirmed pheromones identified in African elephant genital secretions included m-cresol, p-cresol and benzoic acid. Eugenol, a pheromone in swallowtails (*Papilio glaucus*), was also identified (Frankfarter et al. 2009).

**Table 6.4:** Volatile organic compounds (>70% NIST matches), and the class of chemicals they belong to, detected in the labial secretions of adult female African elephants (n=40).

| | Compound | Compound category |
|---|---|---|
| 1 | 1-Heptanol | Alcohol |
| 2 | 1-Hexanol, 2-ethyl- | Alcohol |
| 3 | 1H-Inden-1-ol, 2,3-dihydro- | Alcohol |
| 4 | 1-Nonanol ** | Alcohol |
| 5 | 1-Octanol carbinol ** | Alcohol |
| 6 | 1-Octanol, 5,7,7-trimethyl-2-(1,3,3-trimethylbutyl)- | Alcohol |
| 7 | **1-Octen-3-ol * **** | Alcohol |
| 8 | 1-Pentanol | Alcohol |
| 9 | 2 Ethyl Hexanol | Alcohol |
| 10 | 2-Heptanol, 6-methyl- | Alcohol |
| 11 | 2-Methyl-3-Buten-2-Ol | Alcohol |
| 12 | 3-Buten-2-ol, 2-methyl- | Alcohol |
| 13 | 3-Octanol | Alcohol |
| 14 | Amyl Alcohol | Alcohol |
| 15 | Butenol, methyl- | Alcohol |
| 16 | Ethanol | Alcohol |
| 17 | Isoamylalcohol | Alcohol |
| 18 | Trans-linalool-oxide | Alcohol |
| 19 | 2,6-Dimethylbenzaldehyde | Aldehyde |
| 20 | 3-(4-tertiobutylphenyl)-propanal | Aldehyde |
| 21 | 3-Dodecen-1-Al | Aldehyde |
| 22 | **Benzaldehyde *** | Aldehyde |
| 23 | Benzaldehyde, ethyl- | Aldehyde |
| 24 | **Hexanal *** | Aldehyde |
| 25 | Decane * | Alkane |
| 26 | Dodecane | Alkane |
| 27 | Dodecane, 2-methyl- | Alkane |
| 28 | Hexadecane | Alkane |
| 29 | Hexadecane, 2,6,10,14-tetramethyl- | Alkane |
| 30 | Nonane, 5-(1-methylpropyl)- | Alkane |
| 31 | Octadecane, 2-methyl- | Alkane |
| 32 | Pentadecane | Alkane |
| 33 | Tetradecane | Alkane |
| 34 | Tetradecane, 2-methyl- | Alkane |
| 35 | Tridecane | Alkane |
| 36 | Tritetracontane | Alkane |

| | Compound | Compound category |
|---|---|---|
| 37 | Undecane | Alkane |
| 38 | 1-Decene | Alkene |
| 39 | 1-Heptene | Alkene |
| 40 | 1-Hexene | Alkene |
| 41 | 1-Pentene | Alkene |
| 42 | 1-Pentene, 2-methyl- | Alkene |
| 43 | 2-Methyl-1-acetylcyclopent-1-ene | Alkene |
| 44 | 2-Pentene, (E)- | Alkene |
| 45 | 3-Penten-1-yne | Alkyne |
| 46 | Benzylamne-.Alpha.-D1 | Amine |
| 47 | Hexamine | Amine |
| 48 | N-Ethyl-N-methyl-4-phenetidine | Amine |
| 49 | 1,3-Cyclooctane | Arene |
| 50 | 1,4-Di-n-butylbenzene butylbenzene | Arene |
| 51 | Cyclohexane, 1,2,3-trimethyl-, (1.alpha.2.beta.3.alpha.)-trimethyl-, stereoisomer | Arene |
| 52 | 5-methyl-5-vinyl-1,3-cyclopentadiene | Aromatic compound |
| 53 | 6 Amyl .Alpha. Pyrone | Aromatic compound |
| 54 | Eugenol | Aromatic compound |
| 55 | Pyridine, 2,4,6-trimethyl- * ** | Aromatic compound |
| 56 | 1H-Indene-4-carboxylic acid, 2,3-dihydro-1,1-dimethyl- | Carboxylic acid |
| 57 | Acetic acid * ** | Carboxylic acid |
| 58 | Cyclohexanecarboxylic acid | Carboxylic acid |
| 59 | Heptanoic acid | Carboxylic acid |
| 60 | Pentanoic acid | Carboxylic acid |
| 61 | 2-(1-pentenyl)-furan (Z and E) | Cyclic ether |
| 62 | Furan, 2-pentyl- | Cyclic ether |
| 63 | Trans-2-(1-Pentenyl)Furan | Cyclic ether |
| 64 | 12-Octadecenoic acid, methyl ester | Ester |
| 65 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | Ester |
| 66 | 9-Octadecenoic acid, 12-(acetyloxy)-, methyl ester, [R-(Z)]-ricinoleate | Ester |
| 67 | Amyl Hexanoate | Ester |
| 68 | Benzoic acid, 2-hydroxy-, 3,3,5-trimethylcyclohexyl ester | Ester |
| 69 | **Benzoic acid, methyl ester** * | Ester |
| 70 | **Dodecanoic acid**, methyl ester * | Ester |
| 71 | Ethyl formate | Ester |
| 72 | ethyl hexanoate | Ester |
| 73 | Hexadecanoic acid, methyl ester * ** | Ester |
| 74 | **Hexanoic acid**, methyl ester * ** | Ester |
| 75 | Hexanoic acid, pentyl ester Amyl caproate | Ester |
| 76 | Methyl Hexonate | Ester |
| 77 | Pentadecanoic acid, 14-methyl-, methyl ester * | Ester |
| 78 | Pentanedioic acid, dimethyl ester | Ester |
| 79 | **Tetradecanoic acid**, 12-methyl-, methyl ester * ** | Ester |
| 80 | .Gamma. Hexalactone | Ketones |
| 81 | 2(3H)-Furanone, 5-ethyldihydro- | Ketones |
| 82 | **2-Butanone**, 3-hydroxy- | Ketones |
| 83 | **2-Heptanone*** | Ketones |

| | Compound | Compound category |
|---|---|---|
| 84 | 2-Heptanone, 6-methyl- | Ketones |
| 85 | **3,5-Heptadien-2-one, 6-methyl-, (E)-** | Ketones |
| 86 | 3,7-octadiene-2-one | Ketones |
| 87 | 3-hydroxy-2-butanone | Ketones |
| 88 | 3-methylene-6-hepten-2-one | Ketones |
| 89 | 5-Decanone, 2-methyl- | Ketones |
| 90 | 6-Dodecanone | Ketones |
| 91 | 6-Methyl-3,5-Heptadien-2-One | Ketones |
| 92 | 6-Undecanone ketone | Ketones |
| 93 | Ethanone, 1-(1-cyclohexen-1-yl)- Methyl 1-cyclohexenyl ketone | Ketones |
| 94 | Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)- | Ketones |
| 95 | Ethanone, 1-phenyl- | Ketones |
| 96 | 2-Piperidinone * | Nitro-ketone |
| 97 | 2-Pyrrolidinone .gamma.-Butyrolactam | Nitro-ketone |
| 98 | 1-Dodecanethiol | Organosulfur |
| 99 | **Phenol** | Phenols |
| 100 | Phenol, 2,4-bis(1,1-dimethylethyl)- Phenol, 2,4-di-tert-butyl- | Phenols |
| 101 | Phenol, 2-methoxy- | Phenols |
| 102 | Phenol, 2-methoxy-4-propyl- | Phenols |
| 103 | Phenol, 2-methyl- | Phenols |
| 104 | Phenol, 3,5-bis(1,1-dimethylethyl)- | Phenols |
| 105 | **Phenol, 3-methyl-** * | Phenols |
| 106 | Phenol, 4-ethyl- | Phenols |
| 107 | **Phenol, 4-methyl-** | Phenols |
| 108 | .Delta.-Cadinene | Sesquiterpene |
| 109 | Camphene | Terpenoids |
| 110 | Isoterpinolene | Terpenoids |
| 111 | Limonene* | Terpenoids |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012, 2016), * confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

**6.3.8 Putative chemical characterization of buccal secretions in adult females:** Buccal secretions (Table 6.5) contained more esters than temporal or genital secretions, but had less ketones than temporal or genital secretions, and less phenols than temporal gland secretions (please refer to Table S7.2). Buccal secretions contained a large bouquet of compounds identified as pheromones elsewhere (pherobase.com) – including the sesquiterpenes and terpenoids .alpha.-Humulene, .Beta-caryoplyllene, .gamma.-cadinene, trans-caryophyllene, alloaromadendrene (pheromone in Diptera and Hymenoptera), cyperene (pheromone in Lepidoptera), and aromadendrene. Terpenoids included Limonene and gamma.terpinene – a pheromone in badgers and pandas (Hagey & Macdonald 2003), and in butterflies and a number

of spruce beetles respectively (Francke et al. 1995). Notable furans included Furan, 2,3-dihydro; Furan, 2,5-dihydro-; Furan, 2-pentyl and Furan, 3-butyltetrahydro-2-methyl-,trans-. Buccal secretions shared by the dwarf hamster and African elephant females included acetic acid, pentanoic acid, 9-Octadecenoic acid (Z)-, methyl ester, 9-Octadecenoic acid, methyl ester,- (E) and hexadecanoic acid (Burger 2005).

**Table 6.5:** Volatile organic compounds (>70% NIST matches), and the class of chemicals they belong, detected in the buccal secretions of adult female African elephants (n=40).

| | Compound | Compound category |
|---|---|---|
| 1 | 1,4-Butanediol, 2,3-bis(methylene)- | |
| 2 | 1-Butanol | Alcohol |
| 3 | 1-Butanol, 3-methyl- | Alcohol |
| 4 | 1-Decanol ** | Alcohol |
| 5 | 1-Dodecanol ** | Alcohol |
| 6 | 1-Heptadecanol | Alcohol |
| 7 | 1-Heptanol | Alcohol |
| 8 | 1-Hexanol ** | Alcohol |
| 9 | 1-Hexanol, 2-ethyl- | Alcohol |
| 10 | 1H-Inden-5-ol, 2,3-dihydro- | Alcohol |
| 11 | 1-Nonanol ** | Alcohol |
| 12 | 1-Octanol ** | Alcohol |
| 13 | 1-Octanol, 5,7,7-trimethyl-2-(1,3,3-trimethylbutyl)- | Alcohol |
| 14 | 1-Octen-3-ol ** | Alcohol |
| 15 | 1-Pentanol | Alcohol |
| 16 | 1-Pentanol, 4-methyl- | Alcohol |
| 17 | 1-Tetratdecanol ** | Alcohol |
| 18 | 1-Undecanol ** | Alcohol |
| 19 | 2 Ethyl Hexanol | Alcohol |
| 20 | 2,4-Hexadien-1-ol | Alcohol |
| 21 | 2-Hexyn-1-ol | Alcohol |
| 22 | 2-Methyl-3-Buten-2-Ol | Alcohol |
| 23 | 2-Pentyn-1-ol | Alcohol |
| 24 | 2-Propyldecan-1-ol | Alcohol |
| 25 | 3-Octanol | Alcohol |
| 26 | Amyl alcohol | Alcohol |
| 27 | Butanol | Alcohol |
| 28 | Cis-Linalooloxide* | Alcohol |
| 29 | **Ethanol** | Alcohol |
| 30 | Ethanol, 2-(2-ethoxyethoxy)- | Alcohol |
| 31 | Ethyl Amyl carbinol | Alcohol |
| 32 | Heptanol | Alcohol |
| 33 | Isoamylalcohol | Alcohol |
| 34 | N-octanol | Alcohol |
| 35 | Nonanol | Alcohol |
| 36 | **2 Octanol** | Aldehyde |
| 37 | 2,3-Dimethylbenzaldehyde | Aldehyde |

| | Compound | Compound category |
|---|---|---|
| 38 | 2,4-Nonadienal | Aldehyde |
| 39 | 2,6-Dimethylbenzaldehyde | Aldehyde |
| 40 | 2-Butenal, 3-methyl- | Aldehyde |
| 41 | 2-Heptenal, (E)- * | Aldehyde |
| 42 | 2-Nonenal, (E)- * | Aldehyde |
| 43 | 2-Octenal | Aldehyde |
| 44 | 2-Octenal, 2-butyl- | Aldehyde |
| 45 | 3-(4-tertiobutylphenyl)-propanal | Aldehyde |
| 46 | 4-Pentenal | Aldehyde |
| 47 | **benzaldehyde** | Aldehyde |
| 48 | Benzaldehyde, 3,5-dimethyl- | Aldehyde |
| 49 | Benzaldehyde, ethyl- | Aldehyde |
| 50 | **Decanal** | Aldehyde |
| 51 | Dodecanal | Aldehyde |
| 52 | **Hexanal** | Aldehyde |
| 53 | Hexanal Dimethyl Acetal | Aldehyde |
| 54 | Isophthalaldehyde | Aldehyde |
| 55 | Nonyl Aldehyde | Aldehyde |
| 56 | **Octanal** | Aldehyde |
| 57 | **Pentanal** | Aldehyde |
| 58 | Trans-Nona-2,4-Dienal | Aldehyde |
| 59 | Trans-2-Hexenal | Aldehyde |
| 60 | Undecenal | Aldehyde |
| 61 | Cycloheptane | Alkane |
| 62 | Cycloheptane, methyl- | Alkane |
| 63 | Cyclopropane, 1,1-dimethyl- | Alkane |
| 64 | Cyclopropane, ethyl- | Alkane |
| 65 | Cyclopropane, pentyl- | Alkane |
| 66 | Cyclopropane, propyl- | Alkane |
| 67 | Decane * | Alkane |
| 68 | Decane, 2-methyl- | Alkane |
| 69 | Decane, 3-methyl- | Alkane |
| 70 | Decane, 4-methyl- | Alkane |
| 71 | Dodecane * | Alkane |
| 72 | Dodecane, 2,6,10-trimethyl- | Alkane |
| 73 | Dodecane, 2,6,11-trimethyl- | Alkane |
| 74 | Eicosane | Alkane |
| 75 | Eicosane, 10-methyl- | Alkane |
| 76 | Heneicosane | Alkane |
| 77 | Heptadecane | Alkane |
| 78 | Hexacosane | Alkane |
| 79 | Hexadecane * | Alkane |
| 80 | N-Nonadecane | Alkane |
| 81 | N-Tetradecane | Alkane |
| 82 | Pentacosane | Alkane |
| 83 | pentadecane | Alkane |
| 84 | Pentane, 2-cyclopropyl- | Alkane |
| 85 | Tetradecane * | Alkane |

| | Compound | Compound category |
|---|---|---|
| 86 | Triacontane | Alkane |
| 87 | Tridecane * | Alkane |
| 88 | Undecane | Alkane |
| 89 | Undecane, 5-methyl- | Alkane |
| 90 | 1,3-Pentadiene, (Z)- ( | Alkene |
| 91 | 1-Decene | Alkene |
| 92 | 1-Dodecene | Alkene |
| 93 | 1-Hexene | Alkene |
| 94 | 1-Octene ** | Alkene |
| 95 | 1-Pentene butene | Alkene |
| 96 | 1-Pentene, 2-methyl- | Alkene |
| 97 | 1-Tridecene ** | Alkene |
| 98 | 2,4,6,8-Tetramethyl-1-undecene | Alkene |
| 99 | 2,4-Dimethyl-1-heptene | Alkene |
| 100 | 2-Methyl-1-acetylcyclopent-1-ene | Alkene |
| 101 | 2-Pentene, (E)- | Alkene |
| 102 | 3-Hexene, (Z)- | Alkene |
| 103 | 5-Octadecene, (E)- | Alkene |
| 104 | Cis-Ocimene | Alkene |
| 105 | Copaene | Alkene |
| 106 | Junipene | Alkene |
| 107 | 1-Decyne | Alkyne |
| 108 | 1-Undecyne | Alkyne |
| 109 | Phenylvinylacetylene | Alkyne |
| 110 | 9-Octadecenamide, (Z)- | Amide |
| 111 | Hydroxylamine, O-(2-methylpropyl)- | Amine |
| 112 | Hydroxylamine, O-decyl- | Amine |
| 113 | (-)-Isoledene | Arene |
| 114 | .alpha.-amorphene | Arene |
| 115 | .Alpha.-Copaene | Arene |
| 116 | .Alpha.-Muurolene | Arene |
| 117 | .Alpha.-Pinene, (-)- | Arene |
| 118 | .alpha.-selinene | Arene |
| 119 | .Beta. Bourbonene | Arene |
| 120 | .beta.-cubebene | Arene |
| 121 | .Beta.-Selinene | Arene |
| 122 | 1,3-Cyclooctane | Arene |
| 123 | 1,4-Di-n-butylbenzene | Arene |
| 124 | 1H-Indene, 1-methylene- | Arene |
| 125 | **Azulene** | Arene |
| 126 | Benzocycloheptatriene | Arene |
| 127 | C3-BENZENE | Arene |
| 128 | Ethylbenzene | Arene |
| 129 | germacrene d | Arene |
| 130 | p-Xylene | Arene |
| 131 | (+-)-trans-1-(1-Methylethenyl)-2-(2-methyl-1-propenyl)-cyclopropane propenyl)-, (1R-trans)- | Aromatic compound |
| 132 | (2R*,6R*,8AS*)-6-Hydroxyedulan | Aromatic compound |

| | Compound | Compound category |
|---|---|---|
| 133 | .Alpha.-Humulene | Aromatic compound |
| 134 | **Acetic acid **** | Carboxylic acid |
| 135 | Benzoic acid, 2-mercapto- | Carboxylic acid |
| 136 | Butanoic acid, 3-methyl- | Carboxylic acid |
| 137 | **dodecanoic acid** ** | Carboxylic acid |
| 138 | Heptanoic acid * | Carboxylic acid |
| 139 | Hexadecanoic acid ** | Carboxylic acid |
| 140 | Pentanoic acid | Carboxylic acid |
| 141 | Palmitic acid, * | Carboxylic acid |
| 142 | Pentanoic acid | Carboxylic acid |
| 143 | **Tetradecanoic acid** ** | Carboxylic acid |
| 144 | Oxirane, (1-methylbutyl)- | Cyclic ether |
| 145 | Oxirane, dodecyl- | Cyclic ether |
| 146 | **Furan**, 2,3-dihydro- | Cyclic ether |
| 147 | Furan, 2,5-dihydro- | Cyclic ether |
| 148 | Furan, 2-pentyl- | Cyclic ether |
| 149 | Furan, 3-butyltetrahydro-2-methyl-, trans- | Cyclic ether |
| 150 | 1,1-Dodecanediol, diacetate | Ester |
| 151 | 10,13-Octadecadienoic acid, methyl ester | Ester |
| 152 | 10-Octadecenoic acid, methyl ester | Ester |
| 153 | 11,14,17-Eicosatrienoic acid, methyl ester | Ester |
| 154 | 11-Octadecenoic acid, methyl ester | Ester |
| 155 | 12-Octadecenoic acid, methyl ester | Ester |
| 156 | 13-Octadecenoic acid, methyl ester | Ester |
| 157 | 2-methylpropyl heptanoate | Ester |
| 158 | 6-Octadecenoic acid, methyl ester, (Z)- | Ester |
| 159 | 7-Hexadecenoic acid, methyl ester, (Z)- | Ester |
| 160 | 8,11-Octadecadienoic acid, methyl ester | Ester |
| 161 | 9,11-Octadecadienoic acid, methyl ester, (E,E)- | Ester |
| 162 | 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- | Ester |
| 163 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester * | Ester |
| 164 | 9-Hexadecenoic acid, methyl ester, (Z)- | Ester |
| 165 | 9-Octadecenoic acid (Z)-, methyl ester * ** | Ester |
| 166 | 9-Octadecenoic acid, methyl ester, (E)- * ** | Ester |
| 167 | Allyl Caproate | Ester |
| 168 | Amyl Hexanoate | Ester |
| 169 | Benzoic acid, 2-hydroxy-, 3,3,5-trimethylcyclohexyl ester | Ester |
| 170 | Benzoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester | Ester |
| 171 | Benzoic acid, methyl ester | Ester |
| 172 | Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester | Ester |
| 173 | **Decanoic acid**, methyl ester ** | Ester |
| 174 | **Dodecanoic acid**, methyl ester ** | Ester |
| 175 | Ethyl hexanoate | Ester |
| 176 | Heptadecanoic acid, 16-methyl-, methyl ester | Ester |
| 177 | Heptadecanoic acid, methyl ester | Ester |
| 178 | Heptanoic acid, methyl ester | Ester |
| 179 | Hexadecanoic acid, 14-methyl-, methyl ester | Ester |
| 180 | Hexadecanoic acid, methyl ester * | Ester |

| | Compound | Compound category |
|---|---|---|
| 181 | **Hexanoic acid**, methyl ester * | Ester |
| 182 | Hexanoic acid, pentyl ester | Ester |
| 183 | Homosalate | Ester |
| 184 | Iso Amyl Salicylate | Ester |
| 185 | methyl 10-methylundecanoate | Ester |
| 186 | Methyl 9-Methylundecanoate | Ester |
| 187 | Methyl Decanoate | Ester |
| 188 | methyl dihydromalvalate | Ester |
| 189 | Methyl Dodecanoate | Ester |
| 190 | Methyl Heptanoate | Ester |
| 191 | Methyl Nonanoate | Ester |
| 192 | Methyl tetradecanoate | Ester |
| 193 | Methyl Undecanoate | Ester |
| 194 | Methyl Valerate | Ester |
| 195 | Methylcaproate | Ester |
| 196 | Methyloleate | Ester |
| 197 | Methyl-P-Tert-Butyl Phenyl Acetate | Ester |
| 198 | Nonanoic acid, 9-oxo-, methyl ester | Ester |
| 199 | Nonanoic acid, methyl ester ** | Ester |
| 200 | Octadecanoic acid, methyl ester * | Ester |
| 201 | Octanoic acid, methyl ester ** | Ester |
| 202 | Octyl formate | Ester |
| 203 | Pentadecanoic acid, 14-methyl-, methyl ester | Ester |
| 204 | Pentadecanoic acid, methyl ester ** | Ester |
| 205 | Pentanedioic acid, dimethyl ester | Ester |
| 206 | Pentanoic acid, methyl ester | Ester |
| 207 | Tetradecanoic acid, 12-methyl-, methyl ester | Ester |
| 208 | **Tetradecanoic acid**, methyl ester * ** | Ester |
| 209 | Tridecanoic acid, 12-methyl-, methyl ester | Ester |
| 210 | **Tridecanoic acid**, methyl ester | Ester |
| 211 | Undecanoic acid, 10-methyl-, methyl ester | Ester |
| 212 | Undecanoic acid, methyl ester ** | Ester |
| 213 | Allyl methallyl ether | Ether |
| 214 | .Gamma. Hexalactone | Ketones |
| 215 | 1,3-Cyclopentanedione, 4-butyl- | Ketones |
| 216 | **2-Butanone**, 3-hydroxy- | Ketones |
| 217 | **2-Butanone**, 4-phenyl- | Ketones |
| 218 | 2-Heptanone, 6-methyl- * | Ketones |
| 219 | 2-Hexanone, 5-methyl- * | Ketones |
| 220 | 2-Octanone ** | Ketones |
| 221 | **3,5-Heptadien-2-one, 6-methyl-, (E)-** | Ketones |
| 222 | 3,7-octadiene-2-one | Ketones |
| 223 | 3-Ethylcyclopentanone | Ketones |
| 224 | 3-hydroxy-2-butanone | Ketones |
| 225 | 3-Octen-2-one | Ketones |
| 226 | 4-Formylfuranone | Ketones |
| 227 | 4-Penten-2-one, 4-methyl- | Ketones |
| 228 | 5,9-Undecadien-2-one, 6,10-dimethyl-, (E)- | Ketones |

| | Compound | Compound category |
|---|---|---|
| 229 | 5-Decanone, 2-methyl- | Ketones |
| 230 | 6-Dodecanone | Ketones |
| 231 | 6-Methyl-3,5-Heptadien-2-One | Ketones |
| 232 | 6-Methyl-5-hepten-2-one | Ketones |
| 233 | 6-Undecanone | Ketones |
| 234 | Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)- | Ketones |
| 235 | Ethanone, 1-phenyl- | Ketones |
| 236 | Geranyl acetone * | Ketones |
| 237 | Oxime-, methoxy-phenyl-_ | Nitro compound |
| 238 | 2-Pyrrolidinone | Nitro-ketone |
| 239 | 1-Methyl-5-(4'-methylphenyl)sulfonyl-4-nitroimidazole $ | Organosulfur |
| 240 | tert-Dodecanethiol | Organosulfur |
| 241 | Phenol, 2,4-bis(1,1-dimethylethyl)- | Phenols |
| 242 | Phenol, bis(1,1-dimethylethyl)- | Phenols |
| 243 | α-caryophyllene* | Sesquiterpene |
| 244 | .Beta.-Caryophyllene | Sesquiterpene |
| 245 | .Delta.-Cadinene | Sesquiterpene |
| 246 | .Gamma.-Cadinene | Sesquiterpene |
| 247 | tra**ns-**Caryophyllene***** | Sesquiterpene |
| 248 | Alloaromadendrene | Sesquiterpenoid |
| 249 | Aromadendrene | Sesquiterpenoid |
| 250 | Cyperene | Sesquiterpenoid |
| 251 | y-Terpinene * | Terpenoids |
| 252 | **l-Limonene*** | Terpenoids |

***Bold*** *semiochemical in elephants (Rasmussen 1998, Rasmussen & Krishnamurthy 2000, Goodwin et al. 2002, 2005, 2006, 2012, 2016), *confirmed semiochemical in mammals (Burger 2005). **of mammalian origin (Charpentier et al. 2012).*

Buccal and genital secretions had several alcohols, aldehydes, alkanes, carboxylic acids and ketones in common. Common ketones included gamma. hexalactone; 2(3H)-Furanone, 5-ethyldihydro-; 2-Butanone, 3-hydroxy-; 2-Butanone, 4-phenyl; 3,5-Heptadiene-2-one, 6-methyl; 3,7 Octadiene-2-one; 3-hydroxy-2-butan5-Decanone, 2-mthyl; 5-Dodecanone, 2-methyl; 6-Dodecanone; 6-Methyl-3,5-Heptadien-2-one; 6-Undecanone, ethanone and a nitro ketone 2-Pyrrolidinone. Of the alcohols, 1-Nonanol, 1-Octen-3-ol, 1-Pentanol were found in both buccal and genital secretions. The carboxylic acids found in both secretions included acetic acid, benzoic acid, pentanoic acid, heptanoic acid, tetradecanoic acid (myristic acid), dodecanoic acid (lauric acid) and hexadecanoic acid (palmitic acid). Undecanoic acid, nonanoic acid and heptadecanoic acid occurred only in the buccal secretion.

## 6.4 Discussion:

This study shows for the first time that individual identity is encoded in both buccal and genital secretions, in African elephants. This is confirmed by visual inspection of the chromatograms (Figure 6.1 and Figure 6.2), which showed significant inter-individual variation in compound quality and quantity between individuals, including two highly related sisters, and between four members of the same family unit. These results are supported by prior research on lemurs (*Lemur catta*), where individual identities in labial, scrotal secretions and brachial glands have been described (Scordato et al. 2007). Further studies on marmosets (*Callithrix jacchus*), beavers (*Castor canadensis*), wolverines (*Gulo gulo*), badgers (*Meles meles*) and ferrets (*Mustela furo*) also suggest individual identity is encoded in urine and other specialized scent glands (Smith et al. 2001, Sun Muller-Schwarze 1989, Wood et al. 2009, Service et al. 2001, Zhang et al. 2005)

Results of this research further showed that sex seemed to be encoded in genital secretions (Table 6.1), with statistics tending towards significance. This is supported by other studies that found sex encoded in anogenital gland secretions of the giant panda (*Ailuropoda melanoleuca*), anal glands of otters (Kean et al. 2011), and sternal gland of mandrills (Setchell et al. 2010). It is suggested that, as was the case with TGS and urine, sex is highly likely to be expressed in elephant genital secretions as Asian and African elephants of both sexes can discriminate between musth and oestrus in conspecifics from urine, while males and females track and monitor oestrus from urogenital odour. The insignificant result for sex as a factor may be the result of captured males constituting part of their natal herd (males were calves or juveniles). Contrary to predictions, sex was not encoded in buccal secretions in African elephants. There may be two reasons for this – the first is the absence of sexually mature males. According to Rasmussen (2001), mature Asian bulls' breath contains olfactory signals for musth, and reproductive status, and the absence of mature males may have negated the results. Secondly, it is likely that buccal secretions contain social information pertaining to identity and breeding condition in females, and that sexual signals pertaining to musth and oestrus are more likely to be encoded in penile and labial secretions, urine, dung and TGS. The fact that female adult buccal swabs contained 1-dodecanol, a compound indicating pregnancy,

may be of interest. If confirmed, it would allow for non-invasive sampling of reproductive status in sanctuary environments.

Buccal secretions in African elephants were also found to encode for age (Table 6.1). This result agrees with previous research on a variety of mammals, where age was correlated with chemical profile in rhinos (*Ceratotherium simum*) (Marneweck et al. 2016), pandas (*Ailuropoda melanoleuca*) (Zhang et al. 2008a), mandrills (*Mandrillus sphinx*) (Setchelll et al. 2010), and koalas (*Phascolarctos cinereus*) (Tobey et al. 2009). Age differences were further found to be encoded in male African elephant urine (Rasmussen & Wittemyer 2002). Age class differences are also likely due to differences in diet, gut bacteria and hormonal status, which are known to contribute to odour profiles (Kean & Chadwick 2011).

Finally, “herd” was a significant factor in describing chemical differences between herds, both for genital as well as for buccal secretions (Table 6.1). This was also the case with TGS secretions (Chapter 5). Unexpectedly, neither buccal nor genital secretions were correlated with genetic relatedness at the population, -herd, or - individual level. Herd odour differences may consequently be the result of bacteria. In another fission-fusion mammal that has group-specific social odours, the spotted hyena (*Crocuta crocuta*), bacterial communities sampled from the anal pouches of clan members were more similar to each other than to those from different clans (Theis et al. 2008). This was further supported by studies on meerkats (*Suricata suricatta*) where scent profiles of anal gland secretions were more similar within groups (Leclaire et al. 2017). Elephants exhibit affiliative behaviours among group members, especially during ritualized greeting ceremonies that are likely to enable bacterial transmission from genitalia, mouth, trunk tip, face and gut between unrelated conspecifics. Studies suggest that cross-infection of bacteria arises through frequent physical contact and allo-or-overmarking within groups (Burgener et al. 2008, Theis et al. 2008).

Results indicating that genital secretions encoded for individual identity in this study, and possibly sex, is supported by similar studies on other mammals. Genital secretions have been described in the golden hamster, the giant panda, the common marmoset and the ring tailed lemur. Compounds in genital secretions from these species were found to encode sex differences, age differences, individual identity, fertility and pregnancy (Zhang et al. 2008a,

Smith et al. 2001, Crawford & Drea 2015). In other mammals, vaginal secretions are used for sexual solicitation in for example, golden hamsters, with dimethyl disulfide identified as a major sex attractant (Burger 2005). Semiochemicals identified in the labial secretions of primates, that were also identified in female African elephants, included acetic acid and heptanoic acid (Drea 2014), as well as a number of compounds found in other mammals including benzoic acid, 2-Heptanone, benzaldehyde (Apps et al. 2015). Labial secretions in elephants also had compounds in common with those identified in ring-tailed lemurs and included mainly esters: dodecanoic, hexadecanoic, pentadecanoic and tertradecanoic acids (Scordato et al. 2007). The SIMPER analysis (Table 6.2 and Table 6.3) revealed that acetic acid, hexanoic acid, pentanoic acid, and phenol were important compounds in explaining 30% of the cumulative chemical differences between herds. In fact, acetic and propionic acids may function as a sexual attractant in male chimpanzees (*Pan troglodytes*) (Matsumoto- Oda et al. 2003).

Fatty acids identified by SIMPER – tetradecanoic acid, hexanoic acid and dodecanoic acid, were also identified in elephant male urine by Goodwin et al. (2016) in a seminal study on microbial communities and production of carboxylic acids. Specifically buccal secretions of adult female elephants (Table 6.5) contained similar compounds (Goodwin et al. 2016), including alkan-2-ones and alkan-2-ols such as 2-Heptanone, 2-Tridecanone, octanoic acid, hexanoic acid, decanoic acid, dodecanoic acid and tetradecanoic acid. In the urine of both Asian and African elephants, concentrations of volatile ketones, alcohols, and alkyl phenols increase with time (Apps et al. 2015, Goodwin et al. 2016) suggesting that scent persistence and odour signal intensity is linked to bacterial activity. Urinary compounds identified in the Asian study that were shared in the labial secretion of African elephant females, included ethanol, acetic acid, hexanal and 6-mthyl 3, 5-heptadiene-2-one. 2-Heptanone, a compound suggested to be characteristic of musth urine in Asian elephants (Rasmussen 2001), was also found in adult female African elephants. Of the confirmed African elephant pheromones (Goodwin et al. 1999), m-cresol, and p-cresol were found in labial secretions of adult female African elephants. The SIMPER analysis further identified compounds of significance in describing chemical differences between herds, and these included phenols and acids associated with sexuality and oestrus in other species. Acetic acid is correlated with oestrus, attraction and flight, and m-and-p cresol is linked to

sexuality and oestrus in bison (*Bos bison*) and in horses (Soso 2016). A study on horses (*Equus ferus caballus*) found that both phenols were found in significantly higher concentrations in horse urine during oestrus (Kimura 2001, Buda et al. 2012, Mozuraitis et al. 2012). This is interesting given that the identity of the ovulatory pheromone in African elephants is not known, although it is expected to exist (Bagley et al. 2006) and is suspected to occur in the labial secretions of adult females (Meyer, Goodwin & Schulte 2008).

Buccal secretions in adult elephant females contained an olfactory cocktail of compounds many of which are of mammalian origin, more so than any other elephant odour, and contained more esters than TGS or genital secretions, but fewer ketones than TGS or genital secretions, and less phenols than TGS (Table S7.2). More so than TGS and genital semiochemistry, many compounds identified were of mammalian origin, i.e produced by the elephants themselves. Compounds verified in a previous study on breath in Asian elephant males (Rasmussen 2001) also found in this study, included pentane, 1,3 pentadiene, 2-butanone, acetic acid, hexanal, benzaldehyde, octenal and limonene. Interestingly 2-butanone is an odouriferous ketone that is associated with musth in adult Asian bull, but also with sexuality in lion *Panthera leo*. Additionally, 1-dodecanol was identified in buccal secretions of some adult females, which is an indicator of pregnancy (Charpentier et al. 2012). The esters extracted by SIMPER for herd differences (notably tetradecanoic, dodecanoic, nonanoic, hexadecanoic and decanoic acids) are confirmed pheromones important to the chemical communication among a number of mammals and insects (pherobase.com), and their functional role in African elephants requires further investigation. Compounds identified in the buccal secretions of the dwarf hamster, presumed to be a signal for identity, sex and breeding condition (Singer et al. 1980), were also identified in elephant female TGS (Chapter 5), and in elephant female labial secretions (hexanoic and acetic acids) and buccal secretions (butanoic and pentanoic acids) in African elephants (see SIMPER). Butanoic acid has been identified in anal gland secretions, urine, faeces and hair in canids, primates and rodents (Jones 2017). Nonanoic acid was identified as an important compound in buccal secretions with regards to age and herd differences. This compound has been identified in the urine of leopards, mice and tigers, the anal gland secretions of wolverines and black-backed jackals.

Linoleic acid was found in both secretions, and is a confirmed pheromone in bumblebees, as well as bontebok, blesbok and carnivores such as lion and leopard *Panthera pardus* (Burger 2005). Limonene, an aliphatic hydrocarbon, and a semiochemical in bark beetles (*Scolytinae spp*), honeybees (*Apis mellifera*) and butterflies (Francke et al. 1995) occurred in both secretions, yet buccal secretions contained more sesquiterpenes, sesquiterpenoids and terpenoids in the NIST >70% probability match IDs.

## 6.5 Conclusion

No published reports exist on buccal and genital semiochemistry in wild African elephants. There is thus a need to define key chemical constituents to elucidate possible behavioural function. This research may lead to cross-collaboration with various institutions and conservation agencies in order to verify compounds of interest, test them with behavioural bioassays in the field, and further evaluate the olfactory role of SCFAs in the African elephant's microbiota. Compounds with known biological roles in eliciting behaviour are a good starting point, and specifically those pertaining to sexual signalling, pregnancy and aggression could prove useful in the management of elephants in sanctuary environments.

## 6.6 Limitations:

The results presented here should be interpreted with caution as they reflect a once-off sampling event. Potential confounding factors may include differences in gut flora and behavioural complexity due to the stress of the translocation, as well as complications associated with field research itself with regards to sampling and storage techniques. Identifying the chemical information contained in the secretions of the buccal and genital glands is essential for understanding communication in African elephants, and may hold important implications for managing African elephants both in wild and in captive settings (Schulte et al. 2007).

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# Chapter 7: A pachyderm perfume for sociality in African elephants?

## 7.1 Conclusions

Having evolved over 3.5 billion years, chemical signalling between organisms is the oldest mode of communication between species. Understanding chemical communication and odour-guided behaviour and how it relates to animal behaviour, and how they relate to mate choice, sexual and social behaviour, foraging and territoriality is a complex task requiring a diverse set of skills. Improving the knowledge of African elephant chemical communication will elucidate functional behaviour in this species, and consequently may be of value in navigating elephant conservation and management challenges. In particular, chemical messages conveyed by elephants during greetings, group membership appraisal, individual appraisal, social status appraisals, gender and age appraisal are significant to elephant female sociality, group structuring and competition.

The broad aim of this study was to better understand elephant olfactory communication and specifically, which volatile organic compounds are emitted from urine, TGS, genital and buccal secretions. Given that TGS, genital and buccal secretions have not been described in free-ranging African elephant females, this work provides a novel contribution to behavioural ecology with regards to elephant communication, and provides a platform for further research into the theoretical and practical applications of semiochemicals to the conservation of elephants

Results from this study suggest that African have exceptional olfactory acuity (Chapters 2, 3), and have the ability to differentiate between conspecific urine and dung (Chapter 4). This study explores whether semiochemicals in African elephant urine (Chapter 4), TGS (Chapter 5), buccal and genital secretions (Chapter 6) encoded information about fixed (individual identity, sex, relatedness) and variable (age) information using novel methodologies such as XCMS, and GcAlignR in conjunction with GC_MS and an array of statistical methodologies. For the first time, the chemical composition of temporal gland, buccal and genital secretions in African

elephant adult females is characterised from a wild population. Elucidating the interaction between semiochemistry and behavioural patterns is certain to lead to fresh insights that will be valuable to wildlife managers and conservation practitioners. For example, results from this study reveal that elephant herds differ significantly from each other chemically at the herd level in TGS, buccal and genital secretions, suggesting that herds may share a group odour, but that this odour is not correlated with genetic relatedness. TGS, buccal and genital secretions contained both fixed (sex, individual identity) and variable (age) information, with information differentially expressed in each odour (S7.1). Specifically, results are discussed below with regards to individual recognition, group identity and fission-fusion societies and linear dominance hierarchies in matriarchal societies.

It has long been assumed that TGS, urine and dung released during ritualised greeting ceremonies among African elephant herds are likely to encode individual odour signatures which underlie kin recognition in elephants (Wyatt 2003) and promote bond group cohesion. Consequently, it is feasible too argue that genetic relatedness is expected to correlate to odour to some degree in African elephants. The translocation of 113 elephants in Malawi, with 40 adult females and no adult males, provided an unique opportunity to sample both DNA and TGS, and buccal and genital secretions in a once-off sampling event. Scent mark and body odour is not fixed, as it is influenced by hormones, stress, diet, bacteria and other environmental and physiological factors. The results of this study provide a “window in time” into the semiochemistry of African elephants. Nevertheless, studies suggest that neither diet, bacteria nor environmental factors should mask a true chemical signal of genetic relatedness, suggesting that olfactory cues from odour–gene covariance (OGC) - where chemical similarity covaries with genetic similarity - should reliably reflect genetic relatedness as well as provide signals of biological conditions (e.g. diet) in body odours as a result of the independence between traits, (Raynaud et al. 2009, Archie & Theis 2011).

This study tested whether herds share a group odour, and whether this odour is correlated with genetic relatedness. This expectation was based on the fact that in Amboseli, genetic patterns among elephant herds are important to the inclusive fitness benefits underlying elephant sociality, and that hierarchical structuring was correlated to kin (Archie et al. 2006).

Furthermore, in fission-fusion societies, where females split into multiple subgroups, herd-specific odour may facilitate long-term group stability, as has been confirmed in another clan-living, fission-fusion mammal species, such as the spotted hyena (*Crocuta crocuta)* (Burgener 2008). Genetic relatedness has also been correlated with chemical similarity in other species from primates to rodents (Boulet et al. 2009, Tzur et al. 2009). While “herd” was indeed an important factor in describing differences in chemical profiles for TGSs, and buccal and genital secretions among herds, scent profiles were not correlated with genetic relatedness at the population, herd or individual level (see S7.1). The results were initially surprising, as herd’s fission and fuse along matrilineal and related lines, and odour was expected to play a significant role in kin recognition during fusion, as identifying kin and cooperative partners for social mammals is important to group defence, predator avoidance and offspring care. The assumption that average genetic relatedness would be high among herds as females breed within the group they are born into was not reflected in the data. Research has subsequently suggested that average kinship amongst related females is low in groups with multiple breeding females from successive generations, and polygynous mating systems (Lukas & Clutton-Brock 2018). Further, when the results are viewed and compared with social organization of depredated elephant populations (Wittemyer et al. 2009) with low average genetic relatedness, where hierarchical structuring is not genetically based, and non-relatives comprise groups across social tiers, an olfactory group signal based on genetic relatedness seems extraneous. If herds are comprised of females of varying degrees of relatedness, an olfactory signal for genetic relatedness among social groups would be counter-intuitive. Rather, for sociality to function through the sustained maintenance of cohesive groups, a scent signal for social affiliation, is more likely to exist in herds comprised of related and unrelated females. Affiliative relationships in elephants are maintained not by social grooming as in primates (Dunbar 1988), but through an elaborate system of vocal, visual, chemical and tactile signals and ceremonies between relatives and friends (Moss 1988, Poole & Moss 1989, Poole & Granli 2011). The most well-known of these is the greeting ceremony. Studies have shown that kinship along maternal related lines is not necessary for cooperation and affiliation between families to evolve, as direct benefits of sociality are powerful forces driving the latter. While kinship may be significant for the

evolution of hierarchical social organization, studies on elephants from poached or translocated populations with low average coefficients of relatedness, suggest that elephants maintain hierarchical structure characteristic of elephant sociality (Wittemyer et al. 2009, Gobush et al. 2009). Consequently, this negates the importance of kin-based olfactory signal for social structuring and cohesion among African elephants.

As is the case in elephant bulls (Rasmussen & Krishnamurthy 2000, Slotow et al. 2000), TGS in adult female African elephants may regulate relational complexity among social groups. In the dry season, when resources become limiting, dominance hierarchies emerge in African elephant females and consequently, conflict and competition among group members are likely to occur. It is possible that dominance hierarchies among fission-fusion mammals promote the evolution of traits used in competitive encounters, which are likely to be indirect to minimize injury and guard lifetime reproductive success (Coombes, Stockley & Hurst 2018). Scent marking is often associated with conflict between females over access to resources or reproductive opportunities. Female scent marks, e.g. Major Urinary Proteins in the urine of house mice, have been shown to provide reliable signals of competitive ability that may be used both by competitors and potential mates (Stockley et al. 2013). Direct benefits of sociality can be best optimized through the fluid nature of hierarchical societies, where groups merge and separate in response to familiar patterns. However, signal modalities underlying fusion-fission in African elephants are not well understood. It is possible that adult female odour may, in fact, contain two chemical signals – one signal for fusion and cohesion in the wet season expressed as a “group” odour, and another signalling dominance rank, resulting in fission of bond groups into smaller core units in the dry season. In the dry season resource constraints result in contest competition with dominant females for food and water, which outweigh the direct benefits of sociality. In African elephants, dominance hierarchies are linear and transient, such that older, larger females consistently dominate smaller, younger females (Archie et al. 2006). This has implications for offspring of dominant females, that through their preferential access to resources, may also grow faster and heavier, and are consequently more likely to acquire dominant status as adults. The results from this study, that elephant herds comprised of non-kin share a possible herd odour, supports Hamilton’s model of the evolution of cooperation based

on the concept of inclusive fitness in a fluid fission-fusion society (Hamilton 1964). From an evolutionary perspective, chemical signals underlying possible fission-fusion events among social mammals could pose an elegant and conflict-free solution to mediating the negative effects of increasing group size such as depressed fecundity and increased mortality of females and their offspring (Clutton-Brock, Albon & Guiness 1982).

Group- and individual odour had no correlation and did not covary with genetic relatedness (Odour Gene Covariance), giving rise to the possibility that bacteria may be involved (Odour Bacteria Covariance). Bacteria are proposed to play a significant role in the composition of mammalian odour signals, which contain information about their hosts' genotypes, social and kin relationships. Bacteria, found in host urine, faeces or in products from specialized sebaceous or apocrine scent glands, are suggested to encode information about sex, age, breeding condition, health, diet, dominance rank and social relationships of their hosts. The fermentation hypothesis for chemical communication (Albone et al. 1974; Gorman 1976) suggests that bacteria in specialized scent glands metabolize glandular secretions and so produce volatile odourants that contribute towards mammalian recognition cues. The theory also suggests that olfactory recognition cues are the result of the composition (presence/absence) and structure (abundance of species) of bacterial communities, with some studies suggesting that bacteria also underlie kin-recognition (Archie & Theis 2011). Evidence supporting the fermentation hypothesis comes from studies on short-chain fatty acids (SCFA) in the red fox (*Vulpes vulpes*) and mongooses (*Herpestes auropunctatus*). Both species stopped producing SFCAs with the administration of antibiotics, while cultivated bacteria from the scent glands produced the same SCFA's found in scent marks (Gorman et al.1974, Albone 1978). SCFAS are unlikely to be the only volatile odourants generated by bacteria and a multitude of chemical components in mammalian recognition have been reported (Burger 2005). Group odour has been identified in meerkats and badgers (Leclaire et al. 2017, Gorman et al. 1984). In another fission-fusion mammal that displayed group-specific social odour, the spotted hyena (*Crocuta crocuta*), bacterial communities sampled from the anal pouches of clan members were more similar to those from different clans (Theis et al. 2008). This was supported by studies on meerkats (*Suricata suricatta*) where scent profiles of anal gland secretions were more similar

within than between groups (Leclaire et al. 2017). Studies suggest that cross-infection of bacteria arises through frequent physical contact and allo-or-overmarking within groups (Burgener et al. 2008, Theis et al. 2008). Elephants exhibit frequent affiliative behaviours among group members, especially during ritualized greeting ceremonies that are likely to enable bacterial transmission from genitalia, mouth, trunk tip, face and gut between unrelated conspecifics. Studies suggest that cross-infection of bacteria arises through frequent physical contact and allo-or-overmarking within groups (Burgener et al. 2008, Theis et al. 2008), while recognition of familiar and unfamiliar conspecifics among naked mole rat colonies (*Heterocephalus glaber*) appeared to stem from distinct colony odour labels, contributed by each colony member and distributed among, and learned by, all colony members (O'Riain & Jarvis 1997). Research on bacterial communities in African elephant urine by Goodwin et al. (2016) demonstrated the ability of bacteria to produce alkan-2-ones, and alkan-2-ols, many of which were reported in urinary and buccal odour in this study (Table 6.4 and Table 6.5). The microbiota noted in hyenas and meerkats were noted in these two seminal studies (Theis et al. 2012, LeClaire et al. 2017), and were closely related to the genera Anaerococcus, Anaerovorax, Corynebacterium, Eubacterium, Fastidiosipila, Helcococcus, Porphyromonas and Propionibacterium, and belonged to the bacteria phyla Proteobacteria, Firmiticus, Bacteroietes, Actinobacteria and Fusobacteria. Short chain fatty acids, presumed to be the result of bacterial fermentation and the source of group odour in hyenas and meerkats, were also found in African elephant odour in this study (Table 7.1). The fact that genetic relatedness was not reflected in chemical similarity in this study does not imply that elephants are unable to recognize kin on the basis on their relatedness. Among natural populations of elephants, directing cooperative behaviour to related conspecifics may prove beneficial to cooperative offspring care and preferential resource access. Elephants may use phenotype matching to recognize kin (Archie et al. 2007), and the role of Majorhistocompatability Complex (MHC) derived components or Major Urinary Proteins (MUPs) in elephant urine remain to be fully explored in African elephants. Elephants may also use a combination of modalities, such as acoustic cues, to recognize conspecifics and kin, and may acquire knowledge of the scent of conspecifics through social learning (Stoeger & Baotic 2016, Stoeger & Manger 2014).

**Table 7.1**: Volatile Short Chain Fatty Acids (SFCAs) and Low Molecular Weight (LMW) compounds produced by bacteria in hyenas and meerkats were also found in African elephant secretions.

| | **TGS** | | **Buccal** | | **Genital** | | **Urine** | |
|---|---|---|---|---|---|---|---|---|
| | SIMPER | NIST | SIMPER | NIST | SIMPER | NIST | SIMPER | NIST |
| **SCFAs in hyenas** (after Theis et al. 2012) | | | | | | | | |
| Acetic/Ethanoic acid | x | x | x | x | x | x | | |
| Propionic acid | | | | | | | | |
| Butyric/Butanoic acid | | x | | x | | | | x |
| Isobutyric/ 2-methylpropanoic acid | | | | | | | | x* |
| Valeric/Pentanoic acid | x | x | x | x | x | x | | x |
| Isovaleric acid | | | | | | | | |
| Caproic/Hexanoic acid | x | x | | x | | x | | x** |
| **LMW compounds in meerkats (**after LeCLaire et al 2017) | | | | | | | | |
| 1-hexadecanol | | | | | | | | |
| Lauric/Dodecanoic acid | | | | x | | x | | |
| 1-dodecene | | | | x | | | | |
| Myristic/Tertradecanoic acid | | | | x | | | | |
| **Fatty Acids in elephant male urine (**after Goodwin et al. 2016**)** | | | | | | | | |
| Caproic/Hexanoic acid | x | | | x | x | x | | x |
| Octanoic/Caprilic acid | | | | x | | | | x |
| Capric/Decanoic acid | | | x | x | | | | x |
| Lauric/Dodecanoic acid | | | x | x | | x | | |
| Myristic/Tetradecanoic acid | | | x | x | | x | | |

x* - females only, X** males only, Simper (n= 113), NIST n=40 (adult females only)

A number of volatile organic compounds were identified in African elephants that have a biological role in eliciting behaviour in other mammals (Table 7.2). To better understand elephant chemical communication as it pertains to African elephant social and spatial organization, the >70% NIST matches listed in Chapters 5 and 6 provide a good starting point to select compounds in TGS, genital and buccal secretions for verification with internal standards and subsequent bioassays.

**Table 7.2.** Chemical compounds and their biological role

| Compounds | TGS | Buccal | Labial | Urine | Biological role |
|---|---|---|---|---|---|
| 2-Butanone | | x | x | | Sexuality |
| 2-Pentanone | | | | | Reproduction |
| Dimethyl sulphide | | | | | Sexual Attraction |
| Heptanal | | | | | Aggregation, Excitation, Inhibited behaviour |
| Cyclohexanone | x | | | x | Attraction, stimulation, Inhibition, Locomotion |
| Octanal | | | | | Immobility |
| 2-Nonanone | | | | | Sex attraction |
| Nonanal | | | | | Sexual attraction |
| Acetic acid | | x | x | | Oestrus |
| Benzaldehyde | | | x | x | Defensive, Aggression, Alram recruitment |
| Linalool | x | | | | Alarm recruitment, attraction |
| 1-Octanol | x | x | | | Foraging, Alarm recruitment, sensory perception |
| Acetophenone | | | | x | Attraction |
| Dodecanal | | x | | | Physiological response |
| Phenol | | | x | | Oestrus, Sexuality |
| 4-Methylphenol | x | | x | x | Sexuality, oestrus, Dieostrus, Sexual attraction |
| 3-Methylphenol | x | | x | x | Oestrus |
| isopropyl myristate | | | | | |
| 1-dodecanol | | x | | | Pregnancy |
| 1-hexadecanol | | | | | Bound to sexual pheromone in males |
| Exo-brevicomin | | | | x | Puberty, musth |

*Adapted from Soso & Koziel (2017)*

The compound categories identified in elephant exudates (Table S7.2) are likely to provide information on the persistence and function of the olfactory signal itself. TGS contained more aldehydes than breath or genital secretions, as well as more arenes and aromatic compounds (combined aromatic compounds) than breath or genital exudates, which contained similar amounts. Carboxylic acids were more prevalent in TGS secretions than in breath or genital signals. Breath contained significantly more esters than TGS or genital secretions, yet less phenols than TGS, and ketones were less prevalent in buccal exudates. Aldehydes, prone to oxidation, are commonly found in body odours, rather than scentmarks (Apps et al. 2015), however in Asian elephants, fixatives in TGS yield high molecular weight proteins and lipids and steroids and this high molecular weight ensures signal persistence. Signal longevity in TGS and persistence in a spatially dispersed species such as elephants is important for reproductive success and competitor avoidance for competing males and bulls in search of an oestrus cow. In contrast to urine for example, volatile compounds in glandular secretions appear to

communicate individual traits in some species (Drea 2014). The number of chemical compounds in buccal secretions in this study was considerable, and included many short-chain fatty acids, which are known to reliably discriminate individuals by sex and age (Macdonald et al., 2008). Emanating body odour (genital and buccal secretions) are expected to contain compounds with low molecular weight due to the signal being released in close proximity to conspecifics. So for example, labial secretions in catarrhines included LMW short-chain, aliphatic acids (e.g. acetic, propionic, isobutyric, butyric, isovaleric, and isocaproic acids), which are presumed to arise as byproducts of the fermentation of vaginal flora, and are referred to as 'copulins' as they increase mounting behaviour. Breath is also very rich in chemical compounds, and especially in humans, contains clues to the phase of the gestation cycle, pregnancy and may provide new-borns with a mechanism to recognize their mother (Drea 2014).

## 7.2 Conservation Implications

Conservation objectives can be supported by an improved understanding of how chemical communication in social mammals like elephants influences mate choice, establishing social relationships, courting, mating and the use of scent for establishing territories. In African elephants, an improved understanding of elephant semiochemistry and eliciting behaviour could shed light on scent manipulation for elephant translocation and reintroductions, captive breeding, welfare and enrichment protocols, human elephant conflict (HEC), elephant reintroductions, social behaviour and habitat selection.

1. Population monitoring through use of urine and dung samples at scent marking sites such as waterholes and elephant pathways, could provide information on identity, status, age, sex of an individual and monitor population densities, especially in secretive elephant populations. Research suggests that elephant urine may further contain genetically programmed constituents reflective of relatedness (Rasmussen & Krishnamurthy 2000), and if proven, could prove a useful and non-invasive way of population monitoring. This has proven effective in giant pandas, which have individual specific odour profiles identifiable in anal gland secretions (Zhan et al. 2006).

2. HEC and identification of culprits – recognition and monitoring of known individuals can contribute towards the identification and management of problem elephants and repeat offenders. In Siberian tigers (*Panthera tigris*), sniffer dogs were successfully sued to track specific individuals (Jones 1997) Young male Asian elephants for example, show avoidance behaviour to the TGS scent of musth bulls, retreating from the signal (Rasmussen & Riddle 2004). This is significant as crop-raiding African elephants tend to be young males (von Dürckheim 2011). Further, using learned aversion to for example chilli, predators, and bees, can be used to deter crop-raiding elephants from rural fields.

3. Habitat selection – the cues that elephant use to navigate a foreign landscape post relocation or translocation is of interest. The presence of familiar conspecifics is significant (Stamps 1988) and could be used to encourage departure from release sites or movement along corridors. Elephants deposit scent marks such as urine and dung along pathways, and an improved understanding of what these scent marks encode could be used to manipulate habitat selection and aid in promoting relocation success. Elephants leave TGS, urine and dung deposits at waterholes and along elephant pathways, providing olfactory information about signaller proximity, level of threat of a competitor or the viability of pursuing a fertile mate. Scents could be used to reduce stress response, familiarize residents and stimulate exploration of novel environments post translocation and relocation of elephants, and encourage dispersal and gene flow along corridors in Transfrontier Conservation Areas. Age and sex was encoded in urine, and in TGS (Chapters 4 and 5), allowing elephants to determine age and sex of conspecifics for mate choice and competitor assessment. Elephant males showed a keen interest in female urine (Chapter 2) and are clearly able to track an odour (Chapter 3), while prior research has shown that young males avoid musth bulls and consequently synthetic or translocated musth TGS and urine could be used to restrict movements.

4. Captive welfare - olfactory enrichment can be an important tool in captive animals. Captive breeding programs could benefit from an improved understanding of how odour governs social cohesion and limits competition among conspecifics. Odours in urine, dung or breath, signalling impending ovulation, musth, pregnancy, dominance, or stress, could provide non-invasive tool

to manage a captive population. In the black-footed cat *Felis nigripes,* olfactory cues were used to increase activity and behavioural plasticity, and reducing stress (Wells & Egli 2004).

5. Captive breeding: The introduction of individuals into an established population can result in aggression and/or stress, or initiate physiological response in terms of reproduction, receptivity, and dominance. Further, scent marks containing relatedness markers could be used to monitor levels of hybridization and inbreeding. Olfactory enrichment is important to the welfare of elephants (Schulte et al. 2007) and male and female elephants responded to the scent of unfamiliar conspecifics (Chapter 4) with great interest. The fact that this study supports the role of bacteria in group odour may aid bond group cohesion for newcomers into sanctuary settings, by exposing individuals to secretions from TGS, buccal and genital secretion of the target population prior to translocation to facilitate resettlement. Establishing pregnancy in sanctuary environments could also be facilitated by the identification of an olfactory pregnancy biomarker – isobutyric acid in urine (Chapter 4) and 1-dodecanol in buccal secretions (Chapter 5) may prove good starting points for exploration. Sex-specific odours can be utilised for activating sexual motivation in both sexes as was shown in giant pandas (Swaisgood et al. 2000). From personal observations recorded in this study, the nulliparous female in captivity responded highly aggressively towards the scent of an unfamiliar female, while she reacted with great excitement to the scent of an unfamiliar male. Understanding the effect of scent introductions on same-sex conspecifics is important to manage stress response and monitor behaviour as competition in sanctuaries revolves around access to food and mates. It is feasible to encourage mating with a particular genetically viable yet unfamiliar male, by familiarizing the female to his scent prior to mating introductions.

6. Translocation and relocation programs – semiochemicals that promote social stability are of interest. One study showed that the presence of old mature musth bulls controls the behaviour of younger rogue males (Slotow et al. 2000). It is as of yet unclear whether semiochemicals in female exudates provide a similar function.

7. Health status and disease – stress in the form of salivary Immunoglobin A, fecal glucocorticoid metabolite (fGCM) levels can be monitored in captive and free-ranging elephants in order to manage stress in closed small systems such as sanctuaries and zoos, as well as in

TFCAs in terms of landscape planning and human disturbance. Research into early detection methods for tuberculosis, haemorrhagic septicaemia, trypanosomiasis, pyroplasmosis, foot and mouth disease, pox, bacillary necrosis, salmonellosis, streptococcosis, babesiosis, helminthiasis and ectoparasitism, rabies, tetanus, Anthrax, and Elephant endotheliotropic herpesviruses (EEHV), and their link to expression in elephant odour, would be of value.

## 7.3 Limitations:

The preliminary results presented here should be interpreted with caution as they reflect a once-off sampling event from a translocation targeting family groups. Potential confounding factors may include differences in gut flora and behavioural complexity, also due to the stress of the translocation.

The lack of adult males also impacted the results for specifically genital signals for age sex, as males in this study comprised juveniles and calves. Hormones levels of these young males do not reflect those of fully reproductive musth bulls.

With regards to the chemometric processing, although we identified a large number of semichemical compounds, only compounds soluble in GC_MS were extracted. Lastly, some substances may have further metabolized post sampling, so some substances are likely to be breakdown products.

However, it is hoped that the preliminary results presented here will provide a framework for future research on African elephant adult female semiochemicals, chemical communication and behaviour and contribute to this endangered mammal's conservation and management both in natural and in sanctuary settings.

## 7.4 Future research:

Certain GC_MS NIST compound identifications, if verified by internal standards, would challenge some of the current semiochemical literature in elephants. Additionally, compounds identified in African elephant adult females previously associated with Asian elephant bulls (2-heptanone, 2-butanone, cyclohexanone) require verification by robust replicate sampling. If confirmed by the addition of standards, signalling roles may need to be re-evaluated in the

scientific literature for both Asian and African elephants, in particular with regards to male-male, male-female, and possibly female-female reproductive signalling. Novel compounds among the aromatic compounds, carboxylic acids, sesquiterpenes, sesquiterpenoids and terpenoids should elicit research interest given the convergent evolution of pheromones among Lepidoptera, Coleoptera, Hymenoptera and African elephant semiochemicals reported in earlier studies.

Compounds extracted by SIMPER would be a good starting point for future research into surveying the microbiomes in African elephants using next-generation sequencing methods, and to explore the functional role of SFCAs, and possible pheromones. Further, to understand the significance of results, behavioural bioassays are required to examine the functional role of important compounds identified here to the behaviour of adult females. Specifically, the oestrus signalling potential of m-and p-cresol in labial secretions should be evaluated with behavioural bioassays in African elephants. Also, given that breath encodes pregnancy in humans, 1-dodecanol as a marker of pregnancy in elephants could be explored.

Chemical signals that vary with seasons and in response to resource availability should be investigated in elephant herds, specifically social odour, and dominance rank odour, which serve to underpin fusion and fission respectively. A good starting point may be the three scents that are expressed during greeting ceremonies – TGS, dung and urine. Behaviourally, the body language (ear fanning) suggests that TGS may contain pheromones and scent signals for dominance rank, and this should be further investigated.

Future research could further focus on elephant urinary signal for genetic relatedness given the preliminary results from the sanctuary elephants, reports on Asian elephant calves and as midldly significant result for the correlation between labial secretions and relatedness among highly related (QG>0.45) adult sisters. Further the role of the MHC and MUP in contributing towards odours of genetic relatedness, should be explored (Archie et al. 2007).

Sanctuary environments such as the South African RHCRU and Elephant Whispers, provide excellent opportunities to assess the role of genetics, hormones, bacteria and olfactory profiles of scent signals in African elephants across sexes, ages and reproductive condition.

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# Appendices

## CHAPTER 4

**Table S 4.1** Relatedness was tested using Queller & Goodnight *r* estimate in Coancetsry using 18 microsatellite loci

| | Mussina | Nuanedi | Shan | Chova | Chishuru | Tembo | Medwa | Shamwari | Zizi | Andile | Lindiwe |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mussina | 0.00 | | | | | | | | | | |
| Nuanedi | 0.61 | 0.00 | | | | | | | | | |
| Shan | 0.14 | 0.08 | 0.00 | | | | | | | | |
| Chova | 0.15 | 0.19 | 0.10 | 0.00 | | | | | | | |
| Chishuru | 0.46 | 0.19 | 0.24 | 0.25 | 0.00 | | | | | | |
| Tembo | 0.10 | -0.05 | -0.08 | 0.15 | 0.03 | 0.00 | | | | | |
| Medwa | -0.02 | 0.18 | -0.11 | -0.14 | -0.13 | -0.05 | 0.00 | | | | |
| Shamwari | 0.24 | 0.36 | 0.21 | 0.05 | -0.01 | -0.10 | 0.09 | 0.00 | | | |
| Zizi | -0.04 | 0.20 | -0.23 | 0.05 | 0.06 | -0.28 | 0.26 | 0.31 | 0.00 | | |
| Andile | 0.01 | -0.06 | -0.05 | 0.25 | -0.12 | 0.00 | -0.03 | 0.00 | 0.03 | 0.00 | |
| Lindiwe | -0.08 | -0.20 | -0.23 | 0.09 | -0.03 | -0.12 | -0.07 | -0.16 | 0.03 | 0.17 | 0.00 |

| | |
|---|---|
| >0.50 | A relatedness value of > 0.5 indicates that the animals are very closely related and probably share one or both parents. |
| >0.25 | A relatedness value of > 0.25 indicates that the animals are closely related and probably as half siblings or have a close common ancestor like a grandparent. |
| <0.20 but >0.0 | A relatedness value of >0 but <0.2 indicates that the animals are related but distant relatives. |
| < 0 | A value of <0 indicates that the animals are unrelated. |

**Table 4.2.3** Elephant sex and age of the outgroup elephants

| Name | Sex | Age |
|---|---|---|
| Tembo | Male | 35 |
| Zizi | Male | 22 |
| Shamwari | Male | 24 |
| Medwa | Male | 22 |
| Andile | Female | 22 |
| Lindiwe | Female | 17 |

**Table S4.2:** XCMS extracted 29 urinary VOCs at p <0.01 between male and female adult African elephants.

| | Name | Fold | Log2fold | tstat | pvalue | qvalue | updown | mzmed | rtmed | Female | Male |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M50T17_4 | 6.01 | -2.59 | -64.24 | 0.0002 | 0.28 | DOWN | 50.06 | 16.75 | 2 | 0 |
| 2 | M106T17_3 | 2.37 | 1.24 | 21.46 | 0.0009 | 0.28 | UP | 106.13 | 17.50 | 0 | 2 |
| 3 | M43T27 | 1.17 | -0.22 | -13.55 | 0.0010 | 0.28 | DOWN | 43.04 | 27.17 | 3 | 2 |
| 4 | M457T18_2 | 12.89 | -3.69 | -10.96 | 0.0016 | 0.28 | DOWN | 457.10 | 18.34 | 2 | 0 |
| 5 | M437T45_1 | 4.55 | 2.19 | 12.35 | 0.0017 | 0.28 | UP | 437.03 | 44.95 | 0 | 2 |
| 6 | M121T17_2 | 10.23 | -3.35 | -10.91 | 0.0019 | 0.28 | DOWN | 121.09 | 16.73 | 3 | 0 |
| 7 | M550T22_2 | 4.45 | 2.15 | 10.88 | 0.0021 | 0.28 | UP | 549.83 | 21.73 | 0 | 1 |
| 8 | M488T21_1 | 1.59 | 0.67 | 9.37 | 0.0027 | 0.28 | UP | 488.09 | 21.44 | 1 | 1 |
| 9 | M167T18 | 4.74 | -2.25 | -10.05 | 0.0029 | 0.28 | DOWN | 167.16 | 17.61 | 3 | 0 |
| 10 | M123T22_2 | 6.28 | -2.65 | -9.09 | 0.0031 | 0.28 | DOWN | 123.13 | 21.96 | 3 | 0 |
| 11 | M478T19 | 3.10 | 1.63 | 12.64 | 0.0032 | 0.28 | UP | 477.84 | 19.28 | 0 | 1 |
| 12 | M109T22 | 6.21 | -2.63 | -9.07 | 0.0037 | 0.28 | DOWN | 109.12 | 21.96 | 2 | 0 |
| 13 | M164T17 | 31.76 | -4.99 | -13.76 | 0.0042 | 0.28 | DOWN | 164.12 | 16.73 | 3 | 0 |
| 14 | M152T22 | 6.62 | -2.73 | -9.09 | 0.0045 | 0.28 | DOWN | 152.12 | 21.96 | 3 | 0 |
| 15 | M137T22 | 7.25 | -2.86 | -8.89 | 0.0046 | 0.28 | DOWN | 137.09 | 22.03 | 3 | 0 |
| 16 | M499T20_2 | 4.07 | 2.03 | 7.84 | 0.0047 | 0.28 | UP | 499.39 | 20.20 | 0 | 1 |
| 17 | M524T46_4 | 3.53 | -1.82 | -9.70 | 0.0051 | 0.28 | DOWN | 524.47 | 46.16 | 3 | 0 |
| 18 | M37T17_1 | 1.69 | -0.76 | -8.19 | 0.0052 | 0.28 | DOWN | 37.05 | 17.44 | 3 | 2 |
| 19 | M426T45_1 | 9.75 | 3.29 | 8.27 | 0.0054 | 0.28 | UP | 425.58 | 45.35 | 0 | 1 |
| 20 | M285T46_1 | 5.64 | 2.50 | 8.32 | 0.0054 | 0.28 | UP | 284.55 | 46.12 | 0 | 1 |
| 21 | M65T17_4 | 6.52 | -2.71 | -23.67 | 0.0066 | 0.28 | DOWN | 65.08 | 16.75 | 2 | 0 |
| 22 | M105T10 | 4.38 | -2.13 | -9.48 | 0.0068 | 0.28 | DOWN | 105.13 | 9.82 | 3 | 0 |
| 23 | M488T19_1 | 5.47 | 2.45 | 7.06 | 0.0068 | 0.28 | UP | 487.65 | 18.95 | 0 | 2 |
| 24 | M538T44_1 | 50.47 | 5.66 | 22.29 | 0.0068 | 0.28 | UP | 537.78 | 43.85 | 0 | 1 |
| 25 | M515T45_1 | 7.99 | 3.00 | 7.42 | 0.0068 | 0.28 | UP | 514.56 | 44.98 | 0 | 2 |
| 26 | M50T17_2 | 1.68 | -0.75 | -6.83 | 0.0074 | 0.28 | DOWN | 50.05 | 17.44 | 3 | 2 |
| 27 | M109T7 | 40.99 | -5.36 | -11.19 | 0.0075 | 0.28 | DOWN | 109.09 | 6.77 | 3 | 0 |
| 28 | M63T17_2 | 1.66 | -0.73 | -7.22 | 0.0077 | 0.28 | DOWN | 63.08 | 17.44 | 3 | 2 |
| 29 | M61T17 | 1.71 | -0.77 | -7.59 | 0.0088 | 0.28 | DOWN | 61.05 | 17.44 | 3 | 2 |

**Table S4.3:** Compound categories in African elephant urine

| Compound category | Shan | | Mussina | | Nuanedi | | Chova | | Chishuru | | Zambezi | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | # | % | # | % | # | % | # | % | # | % | # | % |
| Alcohol | 3 | 2.65 | 3 | 3.53 | 5 | 4.59 | 3 | 3.00 | 37 | 32.17 | 21 | 12.21 |
| Aldehyde | 3 | 2.65 | 1 | 1.18 | 3 | 2.75 | 2 | 2.00 | 1 | 0.87 | 0 | 0.00 |
| Alkane | 7 | 6.19 | 5 | 5.88 | 5 | 4.59 | 11 | 11.00 | 6 | 5.22 | 13 | 7.56 |
| Alkene | 11 | 9.73 | 6 | 7.06 | 12 | 11.01 | 13 | 13.00 | 10 | 8.70 | 7 | 4.07 |
| Alkyne | 0 | 0.00 | 0 | 0.00 | 1 | 0.92 | 0 | 0.00 | 0 | 0.00 | 2 | 1.16 |
| Amide | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 1 | 1.00 | 0 | 0.00 | 0 | 0.00 |
| Amine | 2 | 1.77 | 0 | 0.00 | 0 | 0.00 | 1 | 1.00 | 0 | 0.00 | 0 | 0.00 |
| Arene | 16 | 14.16 | 13 | 15.29 | 20 | 18.35 | 31 | 31.00 | 16 | 13.91 | 14 | 8.14 |
| Aromatic compound | 1 | 0.88 | 1 | 1.18 | 1 | 0.92 | 2 | 2.00 | 0 | 0.00 | 1 | 0.58 |
| Carboxylic acid | 0 | 0.00 | 0 | 0.00 | 1 | 0.92 | 0 | 0.00 | 1 | 0.87 | 0 | 0.00 |
| Cyclic ether | 3 | 2.65 | 1 | 1.18 | 1 | 0.92 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| Ester | 6 | 5.31 | 9 | 10.59 | 7 | 6.42 | 1 | 1.00 | 12 | 10.43 | 1 | 0.58 |
| Ether | 31 | 27.43 | 27 | 31.76 | 32 | 29.36 | 23 | 23.00 | 9 | 7.83 | 44 | 25.58 |
| Halide | 1 | 0.88 | 1 | 1.18 | 1 | 0.92 | 0 | 0.00 | 1 | 0.87 | 0 | 0.00 |
| Ketone | 12 | 10.62 | 9 | 10.59 | 7 | 6.42 | 1 | 1.00 | 7 | 6.09 | 18 | 10.47 |
| Monoterpene | 2 | 1.77 | 3 | 3.53 | 3 | 2.75 | 2 | 2.00 | 2 | 1.74 | 0 | 0.00 |
| Nitro-compound | 3 | 2.65 | 1 | 1.18 | 2 | 1.83 | 1 | 1.00 | 3 | 2.61 | 1 | 0.58 |
| Organosulfur compound | 2 | 1.77 | 0 | 0.00 | 1 | 0.92 | 1 | 1.00 | 1 | 0.87 | 0 | 0.00 |
| Phenol | 5 | 4.42 | 5 | 5.88 | 4 | 3.67 | 4 | 4.00 | 8 | 6.96 | 49 | 28.49 |
| Sesquiterpene | 1 | 0.88 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| Sesquiterpenoids | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| Terpenoid | 4 | 3.54 | 0 | 0.00 | 3 | 2.75 | 3 | 3.00 | 1 | 0.87 | 1 | 0.58 |
| Total number of compounds | 113 | | 85 | | 109 | | 100 | | 115 | | 172 | 0 |

# CHAPTER 5

**Table S5.1.** African elephant sample population demography.

| Age | Sex | Herd | Genotyped Individual | Temporal | Buccal | Genital | Complete | No genetic samples | n=113 |
|---|---|---|---|---|---|---|---|---|---|
| 30+ | f | A | M1 | M1 | M1 | M1 | 1 | 16 | |
| 9 | f | A | M2 | M2 | M2 | M2 | 1 | 33 | |
| 6 | f | A | M3 | M3 | M3 | M3 | 1 | 60 | |
| 11 | f | A | M4 | M4 | M4 | M4 | 1 | 63 | |
| 3 | m | A | M5 | M5 | M5 | M5 | 1 | 100 | |
| 7 | f | a | M6 | M6 | M6 | M6 | 1 | 116 | |
| 5 | f | A | M7 | M7 | M7 | M7 | 1 | 118 | |
| 30+ | f | A | M8 | M8 | M8 | M8 | 1 | | |
| 11 | f | a | M9 | M9 | M9 | M9 | 1 | | |
| 30+ | f | a | M10 | M10 | M10 | M10 | 1 | | |
| 30+ | f | a | M11 | M11 | M11 | M11 | 1 | | |
| 10 | f | a | M12 | M12 | M12 | M12 | 1 | | |
| 8 | m | E | M13 | M13 | M13 | M13 | 1 | | |
| 3 | m | a | M14 | M14 | M14 | M14 | 1 | | |
| 6 | m | D | M15 | M15 | M15 | M15 | 1 | | |
| 1 | f | D | M17 | M17 | M17 | M17 | 1 | | |
| 1 | f | e | M18 | na | M18 | M18 | 0 | | |
| 30+ | f | B | M19 | M19 | M19 | M19 | 1 | | |
| 11 | f | C | M20 | M20 | M20 | M20 | 1 | | |
| 30+ | f | B | M21 | M21 | M21 | M21 | 1 | | |
| 30+ | F | B | M22 | M22 | M22 | M22 | 1 | | |
| 9 | f | D | M23 | M23 | M23 | M23 | 1 | | |
| 6 | f | B | M24 | M24 | M24 | M24 | 1 | | |
| 8 | M | B | M25 | M25 | M25 | M25 | 1 | | |
| 3 | f | B | M26 | M26 | M26 | M26 | 1 | | |
| 7 | f | B | M27 | M27 | M27 | M27 | 1 | | |
| 5 | m | D | M28 | M28 | M28 | na | 0 | | |
| 4 | f | B | M29 | M29 | M29 | M29 | 1 | | |
| 15 | f | D | M30 | M30 | M30 | M30 | 1 | | |
| 3 | m | C | M31 | M31 | M31 | M31 | 1 | | |
| 30+ | f | C | M32 | M32 | M32 | M32 | 1 | | |
| 3 | m | D | M34 | M34 | M34 | M34 | 1 | | |
| 30+ | f | E | M35 | M35 | M35 | M35 | 1 | | |
| 3 | m | B | M36 | na | na | na | 0 | | |
| 11 | f | B | M37 | na | na | na | 0 | | |
| 30+ | f | G | M38 | M38 | M38 | M38 | 1 | | |
| 30 | f | D | M39 | M39 | M39 | M39 | 1 | | |
| 35 | f | C | M40 | M40 | M40 | M40 | 1 | | |
| 1 | m | C | M41 | M41 | M41 | M41 | 1 | | |
| 4 | m | C | M42 | M42 | M42 | M42 | 1 | | |
| 6 | m | C | M43 | M43 | M43 | M43 | 1 | | |
| 9 | m | E | M44 | M44 | M44 | M44 | 1 | | |
| 3 | m | E | M45 | M45 | M45 | M45 | 1 | | |
| 4 | m | E | M46 | M46 | M46 | M46 | 1 | | |
| 8 | f | E | M47 | M47 | M47 | M47 | 1 | | |
| 9 | m | H | M48 | M48 | M48 | M48 | 1 | | |
| 28 | f | G | M49 | M49 | M49 | M49 | 1 | | |
| 35+ | f | H | M50 | M50 | M50 | M50 | 1 | | |
| 4 | m | K | M51 | M51 | M51 | M51 | 1 | | |
| 30+ | f | e | M52 | M52 | M52 | M52 | 1 | | |
| 30+ | f | G | M53 | M53 | M53 | M53 | 1 | | |
| 5 | m | G | M54 | M54 | M54 | M54 | 1 | | |
| 1 | m | K | M55 | M55 | M55 | M55 | 1 | | |
| 28 | f | E | M56 | M56 | M56 | M56 | 1 | | |
| 4 | m | G | M57 | M57 | M57 | M57 | 1 | | |
| 4 | f | E | M58 | M58 | M58 | M58 | 1 | | |
| 30+ | f | E | M59 | M59 | M59 | M59 | 1 | | |
| 1 | m | H | M61 | M61 | M61 | M61 | 1 | | |
| 1 | m | G | M62 | na | M62 | M62 | 0 | | |
| 13 | f | H | M64 | M64 | M64 | M65 | 1 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 35+ | f | O | M65 | M65 | M65 | M65 | 1 |
| 5 | f | G | M66 | M66 | M66 | M66 | 1 |
| 1 | m | G | M67 | na | M67 | M67 | 0 |
| 30+ | f | G | M68 | M68 | M68 | M68 | 1 |
| 30 | f | G | M69 | M69 | M69 | M69 | 1 |
| 1 | f | L | M70 | M70 | M70 | M70 | 1 |
| 30+ | f | H | M71 | M71 | M71 | M71 | 1 |
| 8 | m | H | M72 | M72 | M72 | M72 | 1 |
| 17 | m | H | M73 | M73 | M73 | M73 | 1 |
| 40+ | f | M | M74 | M74 | M74 | M74 | 1 |
| 6 | m | H | M75 | M75 | M75 | M75 | 1 |
| 6 | f | L | M76 | M76 | M76 | M76 | 1 |
| 2 | m | M | M77 | M77 | M77 | M77 | 1 |
| 10 | f | Q | M78 | M78 | M78 | M78 | 1 |
| 30+ | f | H | M79 | M79 | M79 | M79 | 1 |
| 5 | f | K | M80 | M80 | M80 | M80 | 1 |
| 30+ | f | Q | M81 | M81 | M81 | M81 | 1 |
| 28 | f | O | M82 | M82 | M82 | M82 | 1 |
| 5 | m | I | M83 | M83 | M83 | M83 | 1 |
| 30+ | f | I | M84 | M84 | M84 | M84 | 1 |
| 6 | f | I | M85 | M85 | M85 | M85 | 1 |
| 1 | m | I | M86 | na | M86 | M86 | 0 |
| 40+ | f | K | M87 | M87 | M87 | M87 | 1 |
| 4 | m | O | M88 | M88 | M88 | M88 | 1 |
| 8 | f | K | M89 | M89 | M89 | M89 | 1 |
| 28 | f | I | M90 | M90 | M90 | M90 | 1 |
| 6 | m | M | M91 | M91 | M91 | M91 | 1 |
| 30+ | f | Q | M92 | M92 | M92 | M92 | 1 |
| 1 | f | G | M93 | na | M93 | M93 | 0 |
| 7 | f | M | M94 | M94 | M94 | M94 | 1 |
| 3 | m | M | M95 | M95 | M95 | M95 | 1 |
| 8 | m | M | M96 | M96 | M96 | M96 | 1 |
| 6 | m | H | M97 | M97 | M97 | M97 | 1 |
| 15 | f | K | M98 | M98 | M98 | M98 | 1 |
| 3 | m | P | M99 | M99 | M99 | M99 | 1 |
| 28 | f | M | M101 | M101 | M101 | M101 | 1 |
| 10 | f | L | M102 | M102 | M102 | M102 | 1 |
| 40+ | f | L | M103 | M103 | M103 | M103 | 1 |
| 28 | f | M | M104 | M104 | M104 | M104 | 1 |
| 3 | f | Q | M105 | M105 | M105 | M105 | 1 |
| 8 | f | O | M106 | M106 | M106 | M106 | 1 |
| 30+ | f | K | M107 | M107 | M107 | M107 | 0 |
| 30+ | f | Q | M108 | M108 | M108 | M108 | 1 |
| 1 | m | P | M109 | M109 | M109 | M109 | 1 |
| 8 | m | P | M110 | M110 | M111 | M110 | 1 |
| 11 | f | P | M111 | M111 | M111 | M111 | 1 |
| 35+ | f | P | M112 | M112 | M112 | M112 | 1 |
| 3 | f | P | M113 | M113 | M113 | M113 | 1 |
| 30+ | f | P | M114 | M114 | M114 | M114 | 1 |
| 5 | f | Q | M115 | M115 | M116 | M115 | 1 |
| 9 | f | P | M117 | M117 | M117 | M117 | 1 |
| 15 | f | P | M119 | M119 | M119 | M119 | 1 |
| 1 | f | Q | M120 | na | M121 | M120 | 0 |

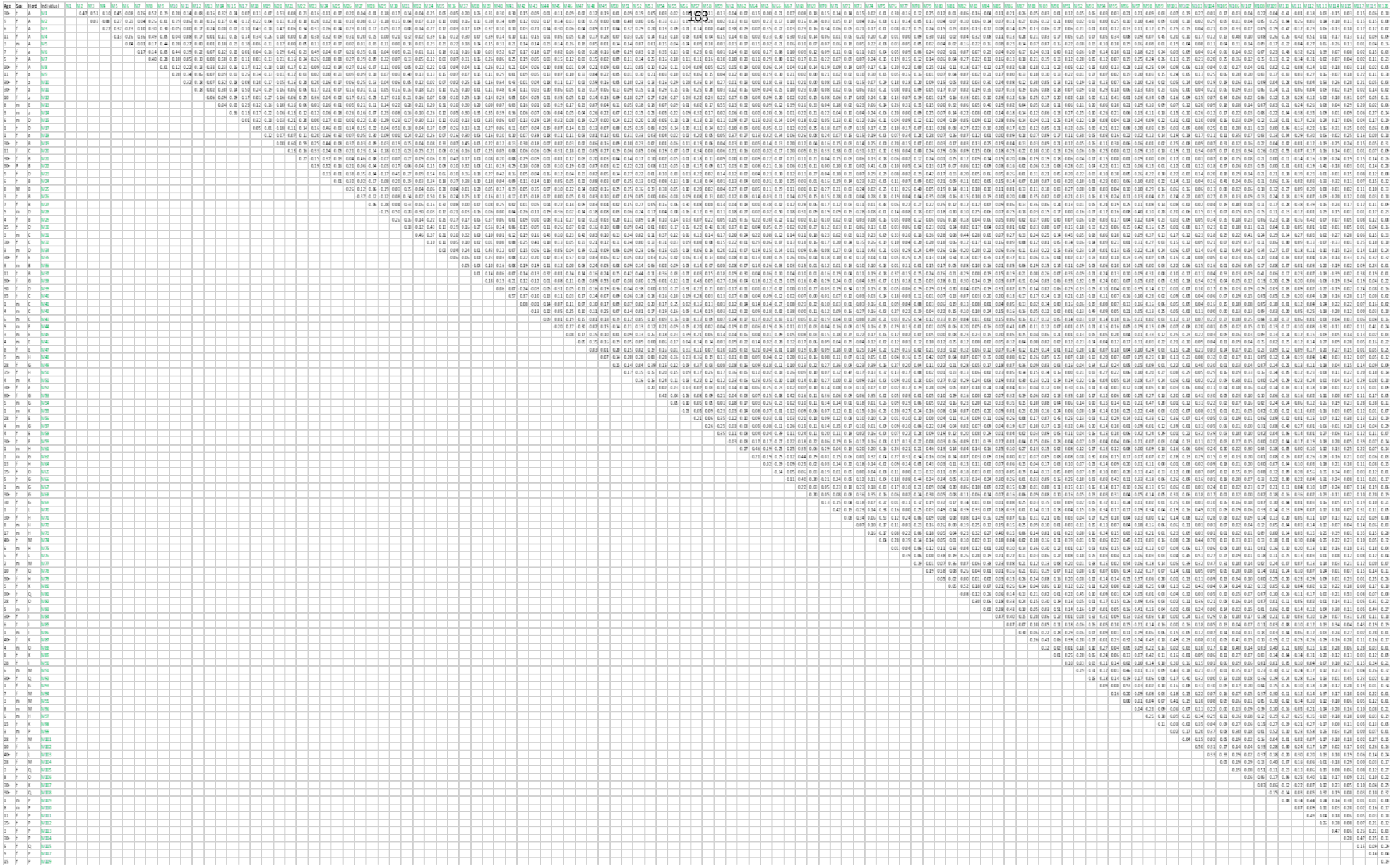

**Figure S5.1:** Queller & Goodnight relatedness estimator *r* values across sample population.

**Table S5.2.** Maternity analysis of elephants - all likely mothers with positive LOD scores listed

95% criteria 80% criteria no assigned mother

| Offspring ID | Candidate mother ID | Pair LOD score | Pair top LOD | Pair confidence |
|---|---|---|---|---|
| M2 | M1 | 5.63E+00 | 5.63E+00 | * |
| M2 | M10 | 3.78E+00 | 0.00E+00 | |
| M2 | M8 | 1.90E+00 | 0.00E+00 | |
| M2 | M69 | 4.37E-01 | 0.00E+00 | |
| M2 | M40 | 3.26E-01 | 0.00E+00 | |
| M3 | M1 | 7.55E+00 | 7.55E+00 | * |
| M3 | M53 | 6.69E-01 | 0.00E+00 | |
| M5 | M1 | 3.90E+00 | 3.90E+00 | * |
| M5 | M10 | 2.80E-01 | 0.00E+00 | |
| M4 | M8 | 1.01E+01 | 1.01E+01 | * |
| M7 | M8 | 5.80E+00 | 5.80E+00 | * |
| M6 | M40 | 7.17E+00 | 7.17E+00 | * |
| M6 | M10 | 1.27E+00 | 0.00E+00 | |
| M6 | M11 | 9.82E-01 | 0.00E+00 | |
| M14 | M10 | 5.59E+00 | 5.59E+00 | * |
| M14 | M40 | 3.86E+00 | 0.00E+00 | |
| M9 | M52 | 3.37E+00 | 3.37E+00 | + |
| M9 | M84 | 1.29E+00 | 0.00E+00 | |
| M12 | M52 | 6.28E+00 | 6.28E+00 | * |
| M37 | M19 | 8.47E+00 | 8.47E+00 | * |
| M26 | M19 | 6.79E+00 | 6.79E+00 | * |
| M27 | M21 | 9.90E+00 | 9.90E+00 | * |
| M36 | M21 | 1.02E+01 | 1.02E+01 | * |
| M24 | M19 | 7.87E+00 | 7.87E+00 | * |
| M24 | M22 | 3.87E+00 | 0.00E+00 | |
| M25 | | | | |
| M29 | | | | |
| M41 | M40 | 5.51E+00 | 5.51E+00 | * |
| M41 | M10 | 4.26E+00 | 0.00E+00 | |
| M41 | M11 | 1.69E+00 | 0.00E+00 | |
| M42 | M40 | 4.85E+00 | 4.85E+00 | * |
| M43 | M32 | 7.58E+00 | 7.58E+00 | * |
| M31 | M32 | 1.09E+01 | 1.09E+01 | * |
| M20 | M1 | 7.78E+00 | 7.78E+00 | * |
| M30 | M39 | 6.12E+00 | 6.12E+00 | * |
| M34 | M39 | 7.16E+00 | 7.16E+00 | * |
| M34 | M30 | 3.08E+00 | 0.00E+00 | |
| M15 | M39 | 9.43E+00 | 9.43E+00 | * |
| M17 | M30 | 9.27E+00 | 9.27E+00 | * |
| M17 | M11 | 2.60E+00 | 0.00E+00 | |
| M17 | M39 | 6.14E-01 | 0.00E+00 | |
| M17 | M59 | 6.10E-01 | 0.00E+00 | |
| M23 | M30 | 6.64E+00 | 6.64E+00 | * |
| M28 | M30 | 8.05E+00 | 8.05E+00 | * |

| | | | | |
|---|---|---|---|---|
| M44 | M35 | 1.45E+01 | 1.45E+01 | * |
| M46 | M35 | 1.56E+01 | 1.56E+01 | * |
| M18 | M52 | 2.66E+00 | 2.66E+00 | + |
| M13 | M59 | 8.10E+00 | 8.10E+00 | * |
| M58 | M59 | 4.51E+00 | 4.51E+00 | * |
| M58 | M104 | 7.93E-01 | 0.00E+00 | |
| M45 | M56 | 5.57E+00 | 5.57E+00 | * |
| M47 | M56 | 5.48E+00 | 5.48E+00 | * |
| M57 | M38 | 7.65E+00 | 7.65E+00 | * |
| M57 | M92 | 3.18E+00 | 0.00E+00 | |
| M57 | M71 | 1.44E+00 | 0.00E+00 | |
| M57 | M49 | 1.04E-01 | 0.00E+00 | |
| M93 | M49 | 8.46E+00 | 8.46E+00 | * |
| M93 | M74 | 1.44E+00 | 0.00E+00 | |
| M54 | M53 | 1.35E+01 | 1.35E+01 | * |
| M54 | M98 | 3.14E+00 | 0.00E+00 | |
| M67 | M53 | 1.07E+01 | 1.07E+01 | * |
| M62 | M68 | 5.80E+00 | 5.80E+00 | * |
| M66 | M68 | 7.58E+00 | 7.58E+00 | * |
| M72 | M50 | 1.10E+01 | 1.10E+01 | * |
| M75 | M71 | 6.70E+00 | 6.70E+00 | * |
| M61 | M79 | 9.18E+00 | 9.18E+00 | * |
| M64 | M79 | 1.01E+01 | 1.01E+01 | * |
| M48 | M79 | 8.38E+00 | 8.38E+00 | * |
| M48 | M10 | 6.22E+00 | 0.00E+00 | |
| M73 | M32 | 3.77E-01 | 3.77E-01 | + |
| M97 | M79 | 9.62E+00 | 9.62E+00 | * |
| M85 | M84 | 6.02E+00 | 6.02E+00 | * |
| M85 | M52 | 2.49E+00 | 0.00E+00 | |
| M86 | M84 | 4.63E+00 | 4.63E+00 | * |
| M83 | M90 | 7.92E+00 | 7.92E+00 | * |
| M55 | M98 | 1.21E+01 | 1.21E+01 | * |
| M89 | M87 | 7.52E+00 | 7.52E+00 | * |
| M89 | M69 | 3.16E+00 | 0.00E+00 | |
| M89 | M98 | 1.97E+00 | 0.00E+00 | |
| M89 | M82 | 1.05E+00 | 0.00E+00 | |
| M51 | M87 | 1.14E+01 | 1.14E+01 | * |
| M51 | M82 | 7.03E+00 | 0.00E+00 | |
| M51 | M98 | 9.97E-01 | 0.00E+00 | |
| M98 | M87 | 9.56E+00 | 9.56E+00 | * |
| M98 | M82 | 3.06E+00 | 0.00E+00 | |
| M98 | M53 | 1.20E+00 | 0.00E+00 | |
| M80 | M82 | 8.13E+00 | 8.13E+00 | * |
| M70 | M53 | 4.09E+00 | 4.09E+00 | * |
| M70 | M103 | 3.87E+00 | 0.00E+00 | |
| M70 | M82 | 7.20E-01 | 0.00E+00 | |
| M76 | M103 | 5.00E+00 | 5.00E+00 | * |
| M76 | M1 | 2.22E+00 | 0.00E+00 | |
| M102 | M103 | 4.29E+00 | 4.29E+00 | * |
| M96 | M74 | 3.75E+00 | 3.75E+00 | + |

| | | | | |
|---|---|---|---|---|
| M104 | M74 | 8.81E+00 | 8.81E+00 | * |
| M104 | M71 | 3.59E+00 | 0.00E+00 | |
| M91 | M101 | 8.44E+00 | 8.44E+00 | * |
| M91 | M87 | 3.27E+00 | 0.00E+00 | |
| M95 | M101 | 7.96E+00 | 7.96E+00 | * |
| M95 | M87 | 3.02E+00 | 0.00E+00 | |
| M95 | M19 | 1.12E+00 | 0.00E+00 | |
| M77 | M104 | 4.12E+00 | 4.12E+00 | * |
| M94 | M84 | 1.28E+00 | 1.28E+00 | + |
| M94 | M11 | 3.62E-01 | 0.00E+00 | |
| M88 | M65 | 5.75E+00 | 5.75E+00 | * |
| M88 | M10 | 2.70E-01 | 0.00E+00 | |
| M106 | M65 | 9.44E+00 | 9.44E+00 | * |
| M106 | M112 | 4.49E+00 | 0.00E+00 | |
| M99 | M112 | 9.59E+00 | 9.59E+00 | * |
| M99 | M65 | 3.70E-01 | 0.00E+00 | |
| M109 | M112 | 4.31E+00 | 4.31E+00 | * |
| M109 | M104 | 2.87E-01 | 0.00E+00 | |
| M111 | M112 | 4.85E+00 | 4.85E+00 | * |
| M113 | M114 | 6.67E+00 | 6.67E+00 | * |
| M113 | M10 | 5.03E+00 | 0.00E+00 | |
| M117 | M114 | 5.03E+00 | 5.03E+00 | * |
| M117 | M59 | 2.37E+00 | 0.00E+00 | |
| M117 | M84 | 1.63E+00 | 0.00E+00 | |
| M119 | | | | |
| M115 | M81 | 8.57E+00 | 8.57E+00 | * |
| M115 | M92 | 4.45E+00 | 0.00E+00 | |
| M120 | M108 | 7.38E+00 | 7.38E+00 | * |
| M78 | | | | |
| M105 | M108 | 8.63E+00 | 8.63E+00 | * |
| M105 | M81 | 5.07E-01 | 0.00E+00 | |

# CHAPTER 6

**Table 6.1** Adult sister dyads with relatedness r estimate of > 0.45 (Queller & Goodnight 1989)

| Herd | Age | Individual | M01 | M08 | 10 | M19 | M21 | M38 | M40 | M49 | M56 | M74 | M81 | M90 | M92 | M103 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 30+ | M01 | 1.00 | 0.52 | 0.20 | -0.07 | 0.08 | 0.26 | 0.30 | 0.19 | 0.08 | -0.02 | 0.01 | 0.01 | 0.05 | 0.27 |
| A | 30+ | M08 | | | 0.12 | -0.10 | 0.17 | 0.11 | 0.12 | 0.09 | 0.04 | -0.17 | -0.11 | -0.22 | -0.12 | -0.18 |
| a | 30+ | M10 | | | | -0.17 | -0.16 | -0.15 | 0.64 | -0.16 | 0.28 | -0.07 | -0.05 | 0.13 | -0.19 | -0.04 |
| B | 30+ | M19 | | | | | 0.60 | -0.05 | -0.12 | -0.16 | 0.11 | 0.25 | -0.17 | 0.21 | -0.05 | 0.09 |
| B | 30+ | M21 | | | | | | 0.00 | -0.20 | 0.03 | -0.01 | 0.13 | -0.25 | 0.18 | -0.04 | 0.01 |
| G | 30+ | M38 | | | | | | | -0.15 | 0.55 | -0.22 | 0.33 | 0.11 | -0.06 | 0.32 | 0.34 |
| C | 30+ | M40 | | | | | | | | -0.07 | 0.19 | -0.03 | -0.13 | 0.13 | -0.15 | 0.04 |
| G | 30+ | M49 | | | | | | | | | -0.09 | 0.39 | -0.21 | -0.03 | -0.04 | 0.40 |
| E | 30+ | M56 | | | | | | | | | | -0.10 | -0.11 | 0.45 | -0.13 | -0.14 |
| M | 30+ | M74 | | | | | | | | | | | -0.01 | 0.11 | -0.01 | 0.45 |
| Q | 30+ | M81 | | | | | | | | | | | | -0.01 | 0.45 | 0.03 |
| I | 30+ | M90 | | | | | | | | | | | | | 0.03 | 0.15 |
| Q | 30+ | M92 | | | | | | | | | | | | | | 0.32 |
| L | 30+ | M103 | | | | | | | | | | | | | | 1.00 |

# CHAPTER 7

**Table 7.1** Odour was not correlated with relatedness at the population, herd, adult sisters and mother-offspring level in African elephants.

| | Genital (n=109) | Buccal (n= 106) | Temporin (n=106) |
|---|---|---|---|
| **ANOSIM** | | | |
| Herd | ANOSIM, global $r^2$ =0.2, p<0.0001*** | ANOSIM, global $r^2$ =0.15, p < 0.0001*** | ANOSIM, global $r^2$ =0.2, p < 0.0019** |
| Age | ANOSIM, global $r^2$ = 0.05 p< 0.16 | ANOSIM, global $r^2$ = 0.06, p< 0.009** | ANOSIM, global $r^2$ = 0.07, p< 0.031* |
| Sex | ANOSIM, global $r^2$ = 0.02, p<0.07 | ANOSIM, global $r^2$ = 1.21, p<0.21 | ANOSIM, global $r^2$ = 1.61, p<0.07 |
| **Spearman Rank Correlation** | | | |
| Population | Mantel's r = 0.006, n=109, P =0.4 | Mantel's r = 0.53, n=106, P = - 0.02 | Mantel's r = 0.02, n=106, P = 0.18 |
| Herd | Mantel's r = - 0.02, n=30, P=0.6 | Mantel's r = -0.024, n=30, P = 0.6 | Mantel's r = -0.024, n=30, P = 0.63 |
| Adult sister dyads | Mantel's r = 0.19, n=14, P=0.04* | Mantel's r = 0.10 n=14, P = 0.16 | Mantel's r = -0.048, n=14, P = 0.67 |
| Mother-offspring dyads | Mantel's r = -0.14, n=18, P= 0.97 | Mantel's r = 0.012, n=18, P = 0.38 | n/a |

**Table 7.2** Major compound categories in TGS, buccal and genital secretions

| | TGS | % | Breath | % | Genital | % |
|---|---|---|---|---|---|---|
| Alcohol | 9 | **10.34** | 35 | **12.20** | 19 | **13.67** |
| Aldehyde | 12 | **13.79** | 25 | 8.71 | 7 | 5.04 |
| Alkane | 6 | 6.90 | 32 | **11.15** | 13 | 9.35 |
| Alkene | 6 | 6.90 | 18 | 6.27 | 7 | 5.04 |
| Alkyne | 0 | 0.00 | 3 | 1.05 | 1 | 0.72 |
| Amide | 0 | 0.00 | 1 | 0.35 | 0 | 0.00 |
| Amine | 1 | 1.15 | 3 | 1.05 | 5 | 3.60 |
| Arene | 4 | 4.60 | 32 | **11.15** | 8 | 5.76 |
| Aromatic compound | 11 | **12.64** | 4 | 1.39 | 9 | 6.47 |
| Carboxylic acid | 9 | **10.34** | 10 | 3.48 | 6 | 4.32 |
| Cyclic ether | 2 | 2.30 | 4 | 1.39 | 3 | 2.16 |
| Ester | 1 | 1.15 | 65 | **22.65** | 17 | **12.23** |
| Ether | 0 | 0.00 | 1 | 0.35 | 0 | 0.00 |
| Halide | 0 | 0.00 | 1 | 0.35 | 0 | 0.00 |
| Ketone | 12 | **13.79** | 25 | 8.71 | 16 | 11.51 |
| Nitro-compound | 2 | 2.30 | 2 | 0.70 | 2 | 1.44 |
| Organosulfur compound | 1 | 1.15 | 2 | 0.70 | 5 | 3.60 |
| Phenol | 7 | 8.05 | 3 | 1.05 | 11 | 7.91 |
| Sesquiterpene | 1 | 1.15 | 5 | 1.74 | 1 | 0.72 |
| Sesquiterpenoids | 1 | 1.15 | 4 | 1.39 | 1 | 0.72 |
| Silane | 1 | 1.15 | 7 | 2.44 | 5 | 3.60 |
| Terpenoid | 1 | 1.15 | 5 | 1.74 | 3 | 2.16 |
| **Total number of compounds** | 87 | | 287 | | 139 | |